



**UNIVERSITY *of*
TASMANIA**

**Studies on Epithelial Mesenchymal Transition
(EMT) in ‘smoking-related’ airway disease**

by

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***Data presented in these papers/abstracts are discussed in the thesis.**

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) is a global health problem, killing approximately 3 million people annually and emerging as the third largest cause of human mortality worldwide. COPD is a poorly reversible chronic, slowly progressive airway obstructive respiratory disease. It is mainly smoking-related and primarily constitutes small airway fibrosis and destruction, more generalized “chronic bronchitis” and in some but not all later development of emphysema. Tobacco smoking is the main causative factor for development of COPD, both in developed and developing countries. Approximately 50% of smokers develop COPD eventually. COPD airways remodeling changes are mostly related to reduce airflow due to small airway fibrosis and ultimately obliteration. Various mechanisms lead to remodeling changes in the airways including perhaps most importantly, dysregulated epithelial basal stem cell function, one feature of which is active EMT. In EMT, epithelial cells transition into a mesenchymal phenotype with subsequent migration into the underlying lamina propria and may be associated with accumulation of myofibroblasts immediately adjacent in the lamina propria. EMT has been implicated in tissue fibrosis in the so called Type-2 form, and when associated with hyper vascularity, it is termed Type-3 which is strongly related to epithelial cancer development.

EMT is a complex process which includes various intracellular pathways including Smad-dependent (TGF β -Smad pathways) and non-Smad dependent pathways (e.g. the Wnt- β catenin and hedgehog pathways), and various nuclear transcription factors (the β -catenin/twist/Snail clusters). It is notable that epithelial cancers make up approximately 90% of all human malignancies suggesting that epithelial cells are especially unstable and EMT- associated mechanisms may be the common factor. Local tissue angiogenesis is especially regarded as another key aspect of both the pre-malignant and malignant phases of epithelial cancer development. Moreover, COPD itself is a major independent risk factor for development of non-small cell (NSC) lung cancer with squamous cell tumors developing in central airways and adenocarcinoma more in the peripheral airway tree. Along with several genetic changes secondary to environmental insult (tobacco smoke) EMT, especially in association with hyper vascularity i.e. Type-3, also has a strong pro-cancerous

influence Thus, the local airway microenvironment is very important in carcinogenesis and metastasis.

Although the expression of EMT biomarkers has been investigated in smokers, the expression pattern of transcription factors and their pathways in COPD has not been reported until now.

Aims: I have investigated the expression of EMT biomarkers in large and small airways, as well as involvement of transcriptional pathway and alterations in transcription factors cellular location. I have investigated smoker with normal spirometry, COPD subjects (both current smokers, CS, and ex-smokers, EX), and compared with normal control tissues. I have also investigated whether EMT biomarker expression in lung cancer is associated with tumor staging and differentiation and how this expression is related to that in the corresponding non-cancerous airway wall.

Methodology: I evaluated the expression of EMT biomarkers (S100A4, Vimentin), an epithelial activation marker (EGFR), and Rbm vascularity (Type-IV collagen) cross-sectionally in both large airways (from resected lung tissue, RT) and small airways (also RT) from: 18 individuals with chronic airflow limitation [combination of smokers with SAD plus frank COPD] and 9 RT control tissues (NC). Differences between peripheral and central area in adeno and squamous cell carcinoma from these same subjects were also evaluated, using the same mesenchymal biomarkers and compared to their expression in large and small airways. I also evaluated the TGF β -Smad pathway and the β -catenin/Snail/Twist transcription factor cluster in large airway bronchial biopsies (EBB) from 15 NLFS, 20 COPD-CS; 15 COPD-EX, and compared these with 15 NC. Anti-S100A4/Vimentin/Type-IV collagen/TGF β -Smad 2/3/7 and anti-transcription factor (β -catenin/Snail/Twist) immunostaining on paraffin-embedded tissue was quantified using computer-aided image analysis.

Results: Large and small airway epithelium showed markedly enhanced expression of S100A4 and vimentin in CAL ($p<0.001$) compared to normal controls (NC). A significant difference in term of increased Rbm vascularity (Type-IV collagen) was observed in large airway (Type-3 EMT) in comparison to small airway (Type-2 EMT)

($p < 0.001$). Small airflow obstruction (FEF25-75%) was correlated significantly with small airway EMT biomarker expression ($R = -0.52$; $p < 0.03$).

In the large airway epithelium, TGF β_1 and pSmad2/3 overexpression was found along with pSmad7 under expression in all clinical groups compared with controls. TGF β_1 expression was especially high in COPD subjects throughout the airway wall ($P < 0.01$), while pSmad 2/3 expression was most marked in the epithelial basal cells and Rbm cells and again especially in current smoking COPD ($P < 0.05$). Expression of inhibitory pSmad-7 was also predominantly reduced in patients with COPD in contrast to smokers and controls ($P < 0.01$). In addition, pSmad but not TGF β_1 expression, was related to airflow obstruction (both FEF25-75% and FER) [($R = -0.74$; $p < 0.001$) for pSmad2/3 and ($R = 0.48$; $p < 0.07$) for psmad7] and the canonical EMT biomarker (S100A4) expression ($R = 0.48$; $p < 0.05$ for pSmad 2/3 and $R = -0.59$; $p < 0.02$ for pSmad7).

An overall increase in β -catenin/Snail/Twist expressing cells in the airway epithelium, Rbm and LP was uniformly found in all clinical groups compared to normal controls along with their significant cytoplasmic to nuclear relocation ($p < 0.05$). However, such changes in Twist expression were not as marked as for the other two transcription factor and mainly significant for NLFS and COPD-Ex ($p < 0.05$). In addition, transcription factors over-expression was significantly correlated not only with airflow obstruction (both FER and FEF25-75%) and EMT activity (S100A4), but also with the TGF β -Smad pathway expression as well.

Significantly increased EMT biomarker expression was found in the peripheral (leading edge) areas of the both adeno carcinoma ($p < 0.001$) and squamous cell carcinoma ($p < 0.001$), in comparison to central areas of these tumors. Moreover, peripheral tumor bio-marker expression was correlated significantly with TNM staging and differentiation score for the whole group of carcinomas, and also with airway epithelial EMT activity. Further, vascularity in the leading edge areas for peripheral squamous cell carcinoma was significantly correlated with large airway Rbm vascularity ($R = 0.68$; $p < 0.05$).

Conclusions: The expression of EMT biomarkers (S100A4 and Vimentin) was upregulated in both small as well as large airways; with EMT categorized on the basis of Rbm vascularity as Type-2 in small airways (pro-fibrotic) and as Type-3 in large airways (pro-malignant as well as pro-fibrotic). In addition, transcription factors (β -catenin/Snail/Twist) and the transcriptional pathway (TGF β -Smad) examined were upregulated in large airways in smokers, but in general, especially so in COPD. These data enhance the strength of evidence for EMT activity being a fundamental part of the pathogenic process of COPD in the airways, and the strong correlations seen between such activity and airway obstruction in especially compelling. Further, increased expression of EMT activity may also be crucial in the pathogenesis of malignancy related to COPD, with “imprinting into the cancer of EMT activity from the already abnormal airway epithelium.

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LIST OF ABBREVIATIONS

| | |
|--------------------------------|---|
| AECOPD | Acute exacerbations of COPD |
| AhR | Aryl hydrocarbon Receptor |
| AIHW | Australian Institute for Health and Welfare |
| ANOVA | Analysis of variance |
| α-SMA | Alpha smooth muscle actin |
| ATS | American Thoracic Society |
| ATT | α 1-antitrypsin deficiency |
| BAL | Broncho alveolar Lavage |
| BASC | Bronchioalveolar Stem cell |
| BHLH | Basic helix-loop-helix |
| BHR | Bronchial hyper responsiveness |
| BMP | Bone morphogenic proteins |
| BOLD | Burden of Obstructive Lung Disease |
| BPD | Broncho pulmonary dysplasia |
| BTS | British Thoracic Society |
| CAF | Cancer associated fibroblast |
| CAL | Chronic airflow limitation |
| CFU | Colony forming unit |
| CigS | Cigarette smoke |
| COPD | Chronic Obstructive Pulmonary Disease |
| COX | Cyclooxygenase |
| CSC | Cancer Stem cell |
| CSE | Cigarette smoke extract |
| CTGF | Connective tissue growth factor |
| DAB | Diaminobenzidine |
| DLco | Diffusing capacity of carbon monoxide |
| DNA | Deoxyribonucleic Acid |
| EBB | Endobronchial biopsy |

| | |
|------------------|--|
| ECM | Extracellular Matrix |
| EGFR | Epidermal Growth Factor Receptor |
| EMT | Epithelial-Mesenchymal Transition |
| ERS | European Respiratory Society |
| FDA | Food and Drug Administration |
| FEF25-75 | Force mid-expiratory flow rate |
| FER | Forced expiratory ratio |
| FEV1 | Forced expiratory volume in one second |
| FGFR | Fibroblast growth factor receptor |
| FSP | Fibroblast specific protein |
| FVC | Forced Vital Capacity |
| GlcNAc | N-Acetylglucosamine |
| GM-CSF | Granulocyte-Macrophage Colony Stimulating Factor |
| GOLD | Global Initiative for Chronic Obstructive Pulmonary Lung Disease |
| GPCR | G-protein coupled receptor |
| GSK-3b | Glycogen synthase Kinase 3 beta |
| H & E | Haematoxylin and Eosin |
| H2O2 | Hydrogen peroxide |
| HAT | Histone Acetyl Transferases |
| HBEC | Human bronchial epithelial cells |
| HCC | Hepatocellular carcinoma |
| HDAC | Histone deacetylases |
| HGF | Hepatocyte growth factor |
| HIF-1a | Hypoxia inducible factor 1 alpha |
| H-N | Healthy Normal |
| HRP | Horseradish Peroxidase |
| ICS | Inhaled Corticosteroids |
| IFN-g | Interferon g |
| Ig | Immunoglobulin |
| IGF | Insulin like growth factor |
| IL | Interleukins |

| | |
|--------------|---|
| IPF | Idiopathic pulmonary fibrosis |
| JNK | Janus Kinase |
| LDLR | Low-density lipoprotein receptor |
| LEF-1 | Lymphoid Enhancing Factor- 1 |
| LP | Lamina propria |
| LPS | Lipopolysaccharide |
| LRP | Lipoprotein receptor related protein |
| LRTI | Lower respiratory tract infections |
| LTB4 | Leukotriene B4 |
| MAPK | Mitogen activated protein complex |
| MHC | Major-histocompatibility-complex |
| miR | MicroRNA |
| MMPs | Matrix metalloproteinases |
| MPO | Myeloperoxidase |
| mRNA | Messenger Ribonucleic Acid |
| nAChR | Nicotine Acetylcholine Receptor |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NC | Normal control |
| NCDs | Non-communicable diseases |
| NF-kB | Nuclear factor kappa B |
| NLFS | Normal lung function smoker |
| NNK | Nicotine derived nitrosamine ketone |
| NO | Nitric Oxide |
| NSCLC | Non-small cell lung cancer |
| OR | Odds ratio |
| PAH | Polycyclic Aromatic hydrocarbons |
| PDGF | Platelet derived growth factor |
| PEF | Peak expiratory flow |
| PI3K | Phosphoinositide-3-kinase |
| PM | Particulate matter |
| PTM | Post transcriptional modification |
| RAGE | Advanced Glycosylation end product receptor |

| | |
|---------------|--|
| Rbm | Reticular Basement Membrane |
| RNA | Ribonucleic Acid |
| ROS | Reactive oxygen species |
| RR | Relative risk |
| RT | Resected tissue |
| RTK | Receptor tyrosine kinase |
| SAD | Small airway disease |
| SE | Standard error |
| SES | Socioeconomic status |
| Shh | Sonic Hedgehog |
| SHS | Second hand smoke |
| SMC | Smooth muscle cells |
| SMO | Smoothened Protein |
| STAT | Signal transducer and activator of transcription |
| SUMO | Small Ubiquitin like modifier |
| TACE | Tumour necrosis factor alpha converting enzyme |
| TAHS | Tasmanian Longitudinal Health Study |
| TAM | Tumour associated macrophages |
| TB | Tuberculosis |
| TCF | T- cell factor |
| TF | Transcription factor |
| TGF-b1 | Transforming growth factor-Beta1 |
| TNF-a | Tumour Necrosis Factor-Alpha |
| UIP | Usual interstitial pneumonia |
| UPAR | Urokinase type plasminogen activator receptor |
| VEGF | Vascular Endothelial Growth Factor |
| WHO | World Health Organization |
| Wnt | Wingless tail |
| ZEB | Zinc finger E-box |
| ZO-1 | Zona Occludin 1 |

Chapter 1

1. Introduction

Being a medical professional and especially as a trained pathologist, for some years I have had a particular interest in respiratory disease and its pathogenesis and pathophysiology. This is the reason that I came to Australia to work in a group that had worked on these very issues for many years, and had recently published papers challenging the current dogmatic concepts of smoking-related airway disease processes, and indeed changing the very understanding of these. They had described active epithelial-mesenchymal transition and epithelial hyper vascularity in large airway biopsies, related to smoking pack-year history, and seemingly having physiological consequences. It was very exciting to have the opportunity to continue with this relatively preliminary work, to take a few new steps “towards the light”, to extend findings to small airways, to define more basic mechanisms, and in the later phase of my PhD research to try and link COPD pathology more closely with non-small cell lung cancer than previously achieved.

I will deal in this chapter, briefly, with an overall conceptual and reductionist “model” of what the facets of COPD pathology consist of (Figure 1.1). This helped me to “place” my research contextually within a broader theme of work going on locally, but which might also help the reader to see where this thesis fits in with the bigger picture.

I will also deal with an outline of what COPD is, from the clinical and physiological angles, what the risk factors are for it, and its epidemiology and its impacts at personal, social and global levels.

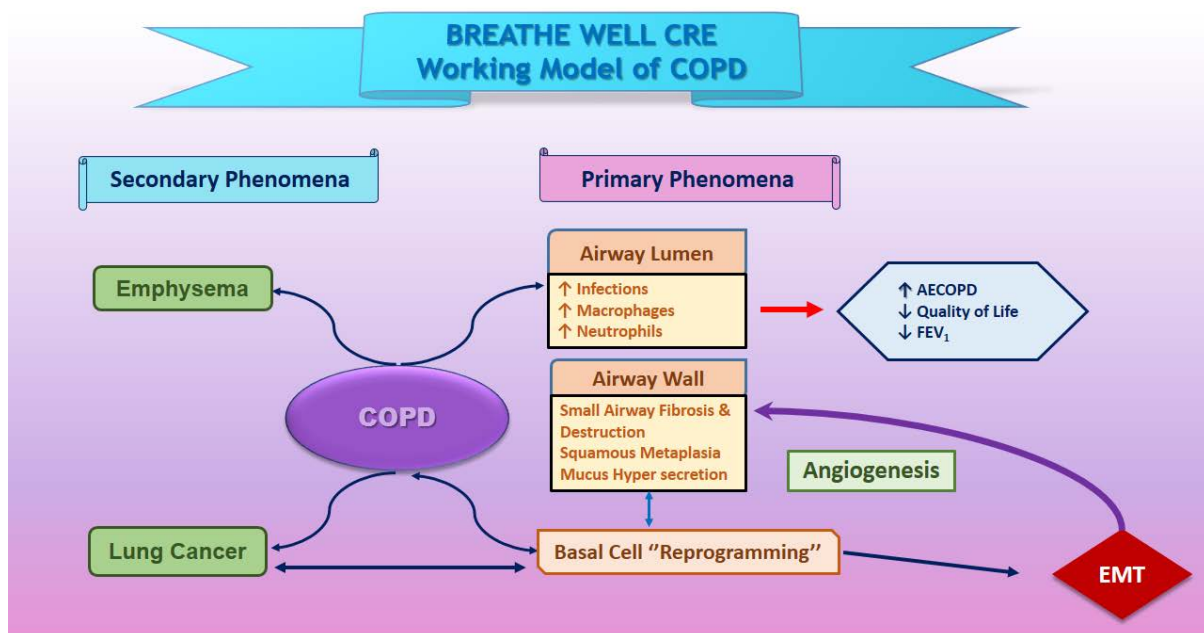


Figure 1-1 Model of COPD

Developed over a decade by researchers within the Breath Well CRE, at the University of Tasmania.

As I will review in greater detail shortly, it has been known for several decades since the pioneer studies of Lynne Reid and Michael Dunnill in England and the team around Peter Macklem in Canada, that the airway epithelium and indeed the mucosa generally was structurally markedly abnormal in smokers and especially in COPD, and that the prime site for airflow obstruction was in the small airways less than 2mm in diameter when the wall was thickened and fibrotic with progressive obliteration of the airway lumen. This is the early and primary pathology in physiological COPD, for which perhaps the term Chronic Airflow Limitation (CAL) would have been better applied; this occurs even before an individual is symptomatic. Work by Ron Crystal's group at Cornell University in New York has quite recently indicated that the fundamental change is likely to be genetic reprogramming of the basal epithelial cells, which are the airway stem cell. In many ways we came to a similar conclusion in a parallel fashion though description of EMT in this very same cell population.

Another early series of events involves the airway lumen; these have really taken the major bulk of research interest in more recent years, and are certainly associated with

symptomatic illness. The key event is activation of the innate immune system, and acute and chronic infection, but which is cause and which effect is not really well worked. Another interest of our CRE coincident with my work has been why *Haemophilus influenza* and *Pneumococci* in particular are the bacterial species involved in COPD, and why Rhinoviruses are also so important in its natural history. The local focus has been on up-regulation of specific surface adhesion sites for these “respiratory” pathogens as part of epithelial dysfunction.

Finally, there are events that are common but not universal, and especially development of emphysema (parenchyma destruction and loss of the elastic recoil properties of the lungs) and lung cancer in many but not all COPD sufferers, and usually one or more decades later than the airway remodeling and inflammatory/infective processes discussed above.

Out of this complex syndrome of real and/or potential disease processes, my research has very much focused on the airway wall, and mechanisms of fibrosis and destructive part of the pathology. This reductive approach I feel is important, because when others say they are studying “COPD” or a putative model of COPD, it may well be a quite different issue and set of questions being addressed, and not infrequently their relative importance and contribution to the overall clinical and pathological picture is quite poorly defined.

Chapter 2

2. **Chronic Obstructive Pulmonary Disease (COPD) and Epithelial mesenchymal transition: Literature Review**

Chronic obstructive pulmonary disease (COPD) is a huge global health problem with increasing morbidity and mortality. COPD is a preventable but only partially treatable disease associated with progressive respiratory disability, hospital admissions for acute exacerbation (AECOPD) and impairment of quality of life. In this literature review I will first deal with the basics of COPD as a clinical entity and then discuss the process of Epithelial-Mesenchymal Transition (EMT) and the potential for this to be a major driving pathogenic process, as a part of a broader “reprogramming” of the airway epithelial basal ‘stem’ cells.

2.1 Anatomy

It is necessary to understand the anatomy of the lungs before proceeding to actual pathogenic events related to the airways in COPD.

Lung comprises two chief anatomical structures: airways and parenchyma. The tracheobronchial tree is a highly branched system of conducting tubes, that can be divided anatomically into large airways or bronchi, (usually >2mm in internal diameter and with supporting cartilage in the airway wall) and small airways or bronchioles <2mm in internal diameter with little no cartilage in their walls (Bohadana, Teculescu & Martinet 2004).

The gas-exchange lung parenchyma consists of respiratory bronchioles, alveolar ducts, alveolar sacs and especially vast number of alveoli. Anatomically the conducting bronchioles are vital as they each serve a pulmonary lobule - the basic unit of the lung (Shapiro, S, Snider & Rennard 2005) (Figure 2.1).

Microscopic imaging of large and medium airways, illustrate that the most distinctive structures are epithelium, basement membrane (BM) and reticular basement membrane (Rbm), the lamina propria (LP) and smooth muscle layer. All of the above together constitutes the mucosa, and then deep to this is the sub-mucosa consisting of cartilage and connective tissue adventitia (Junqueira, Carneiro & Kelley 2005) (Figure 2.2).

The mucosa thus has an epithelial layer on the luminal surface, and this is pseudo stratified in that there are basal cells and fully developed epithelial cells which all attach to the basement membrane. Epithelial cells for the most part are elongated and have cilia on their apical surface, though there is sub-set of mucus secreting “goblet cells” in addition. The true basement membrane (BM), is a thin linear structure composed of collagen IV (Thurlbeck & Churg 1995). BM actually detach the epithelium from the lamina propria (LP) whereas LP lies between the BM and the inner side of the smooth muscle layer. The LP is composed of connective tissue containing cells and blood vessels. The sub-mucosa deep to the smooth muscle also contain mucous glands, cells and loose connective tissue (Thurlbeck & Churg 1995) (Figure 2.2).

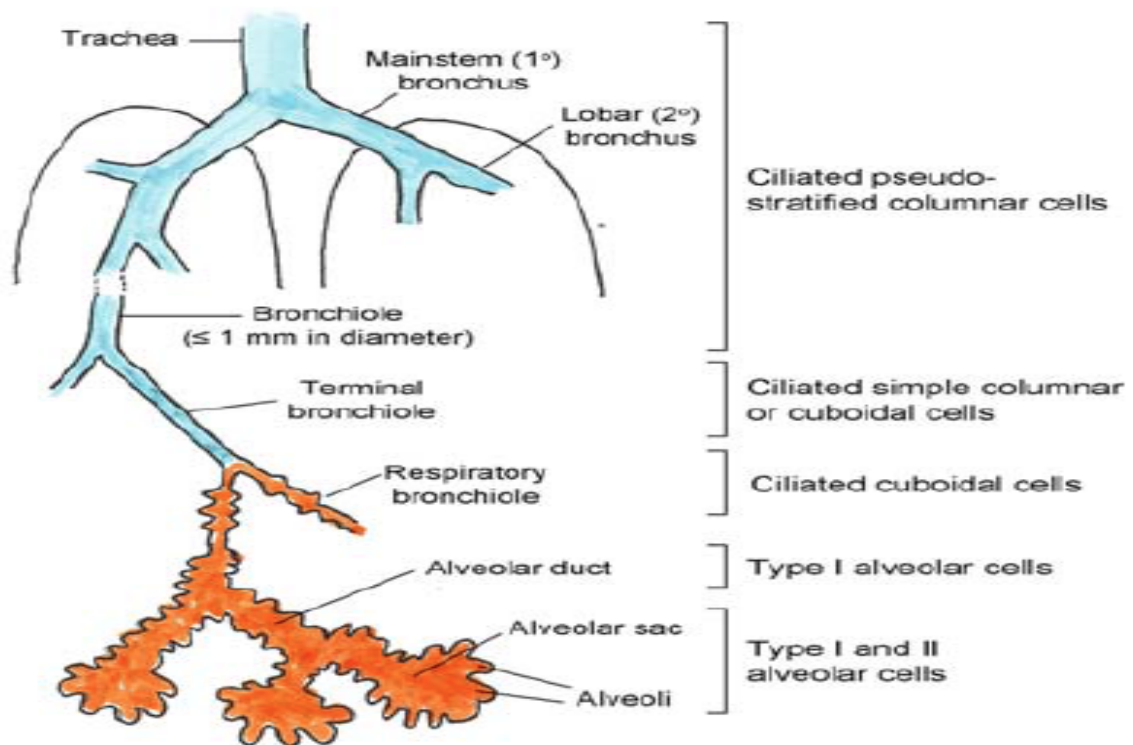


Figure 2-1: Schematic demonstration of central and peripheral airways

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Figure 2-2: Microscopic images of biopsy taken from airway by Fiberoptic Bronchoscopy.

Epithelium (EP), the very superficial layer, is separated from underlying lamina propria (LP) by basement membrane (black arrows). Lamina propria is primarily made up of loose connective tissue and blood vessels. Hardly ever submucosa can be seen in biopsies, which have mucous glands.

2.2 Definition of COPD

According to the pre-eminent Global Initiative for Chronic Obstructive Lung Disease (GOLD), a currently widely used functional definition of COPD is "a disease state characterized by limitation of airflow which is slowly progressive and not fully reversible. This chronic progression of airflow limitation is usually associated with background of an abnormal inflammatory response of the lungs to noxious particles or gases" (GOLD 2015). The concept of the widely accepted 'inflammatory' response as a hallmark of COPD, may in fact not be relevant to the airway wall (Sohal, S, Eapen, MS, Tan, D, Muller, HK & Walters 2015) although it does probably apply to the airway and alveolar lumen.

2.3 Classical pathology of COPD

The "classical" pathology of COPD was described several decades ago, beginning with Lynn Reid in London reporting in 1954, the increased numbers of acini and subsequent thickness of airways mucous glands, recognized as feature of "chronic bronchitis", i.e. chronic cough and sputum production, thought then to be synonymous with COPD. This study standardized now the famous Reid index for describing gland/wall thickness ratio, which she showed was increased in chronic bronchitis. Unfortunately, this lesion of mucous gland hyperplasia was not closely correlated with airflow measurements. So emphasis was mainly on anatomical changes rather than obstructive pathology associated with COPD (Reid, L 1954). In 1967, Macklem and colleagues added insights into peripheral or small airway disease as the site of increased airway resistance in smokers (Macklem & Mead 1967). Thus small airways disease as shown as a potential "silent zone" where pathology can accumulate for a long period of time without being clinically obvious or measurable. (Hogg, Macklem & Thurlbeck 1968) (Figure 2.3).

In 1969, another major pioneering study by Michael Dunnill and colleagues in Oxford showed emphysema, although associated with COPD in smokers in general, occurred decade or later than airway narrowing, and indeed was not invariable even then (Dunnill 1969). In 1985, Snider et al defined emphysema pathologically as " permanent alveolar wall destruction with irreversible enlargement of the air spaces distal to the terminal bronchioles and without evidence of fibrosis" (Snider et al. 1985). Morphologically, there are two main subtypes of emphysema (Kim, WD et al. 1991).

The panacinar form of emphysema is associated with α 1-antitrypsin deficiency, resulting in an even dilatation and destruction of the entire acinus. The most common centriacinar emphysema form, causes more severe small airways obstruction and is mostly associated with cigarette smoking, and without obvious enzyme deficiency (Litmanovich, Boisselle & Bankier 2009) (Figure 2.5). An in depth detail of emphysema is beyond the scope of this thesis.

Large airway involvement in COPD is itself quite complex, with squamous metaplasia of epithelium as well as Reid's "Chronic Bronchitis" changes with or increased cough and sputum production. (Dunnill 1969; Hogg 2004; MacNee 2005) (Figure 2.4). Further details are furnished in the remodelling section, later in this chapter.

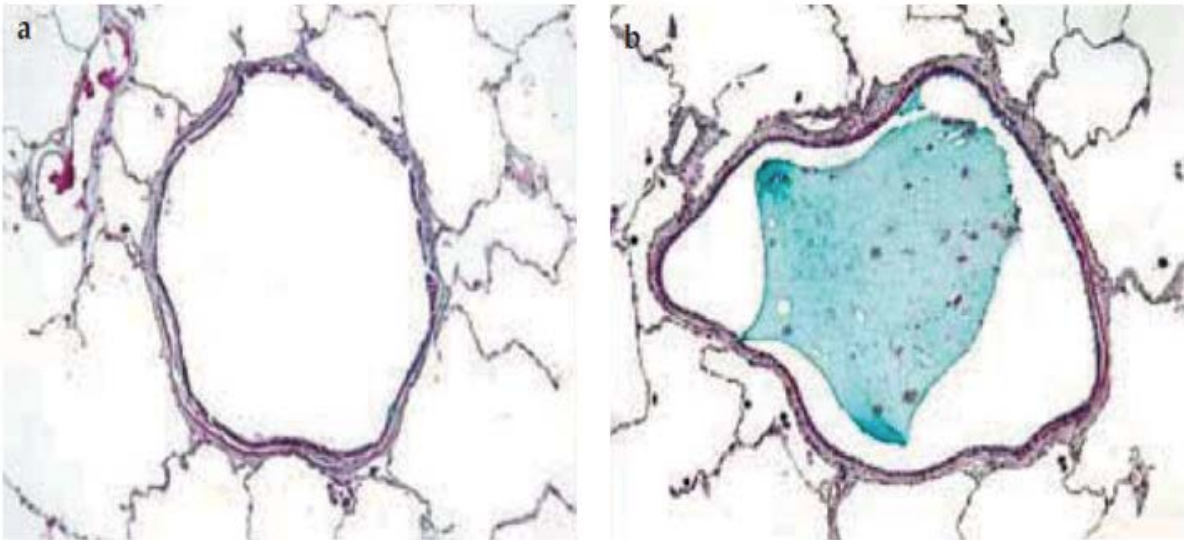


Figure 2-3: Small Airway Obstruction

- a) Normal small airway and alveoli b) thickened small airway with mucus plug filling airway lumen in chronic obstructive pulmonary disease

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Normal Smoker

Chronic Bronchitis

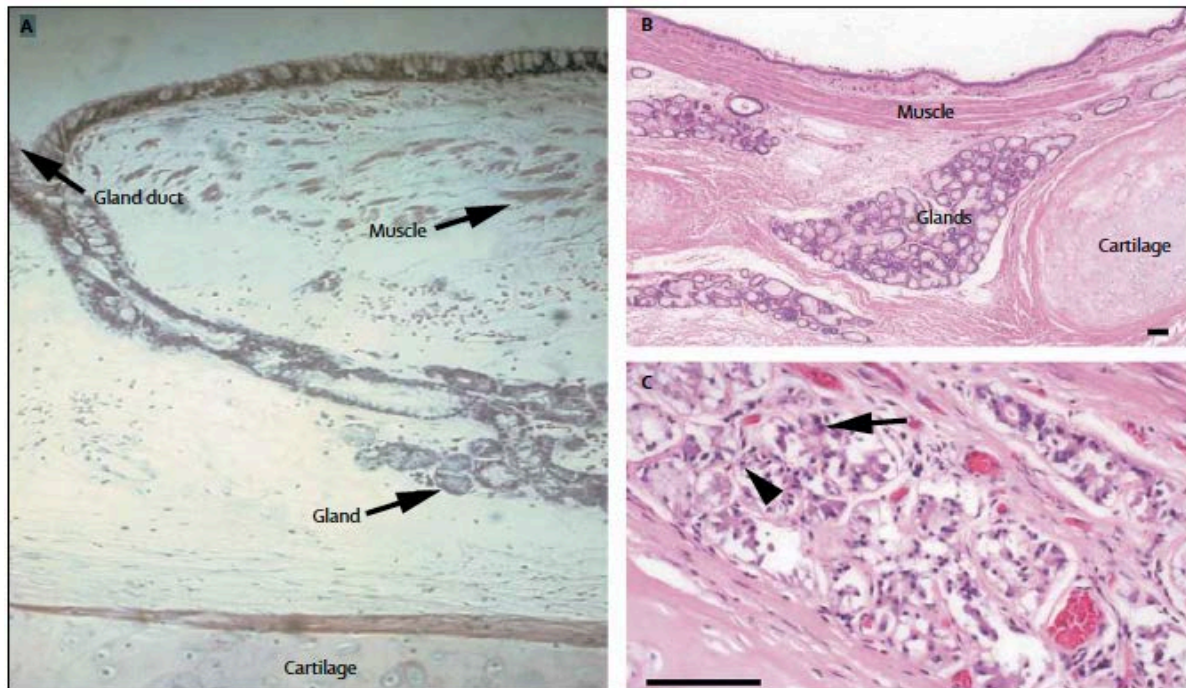


Figure 2-4: Pathologic changes of the central airways in COPD.

Pathologic changes of the central airways in COPD. A central bronchus (A) from the lung of a physiologically normal cigarette smoker, demonstrates the presence of relatively little bronchial smooth muscle and small epithelial glands. In contrast, in subject (B) with “chronic bronchitis”, bronchial smooth muscle is arranged as a thick bundle and drastically enlarged bronchial glands. However, (C) has the enlarged bronchial glands at an advanced level, with localized chronic inflammation involving leukocytes (arrowhead) and mononuclear cells, together with plasma cells (arrow).

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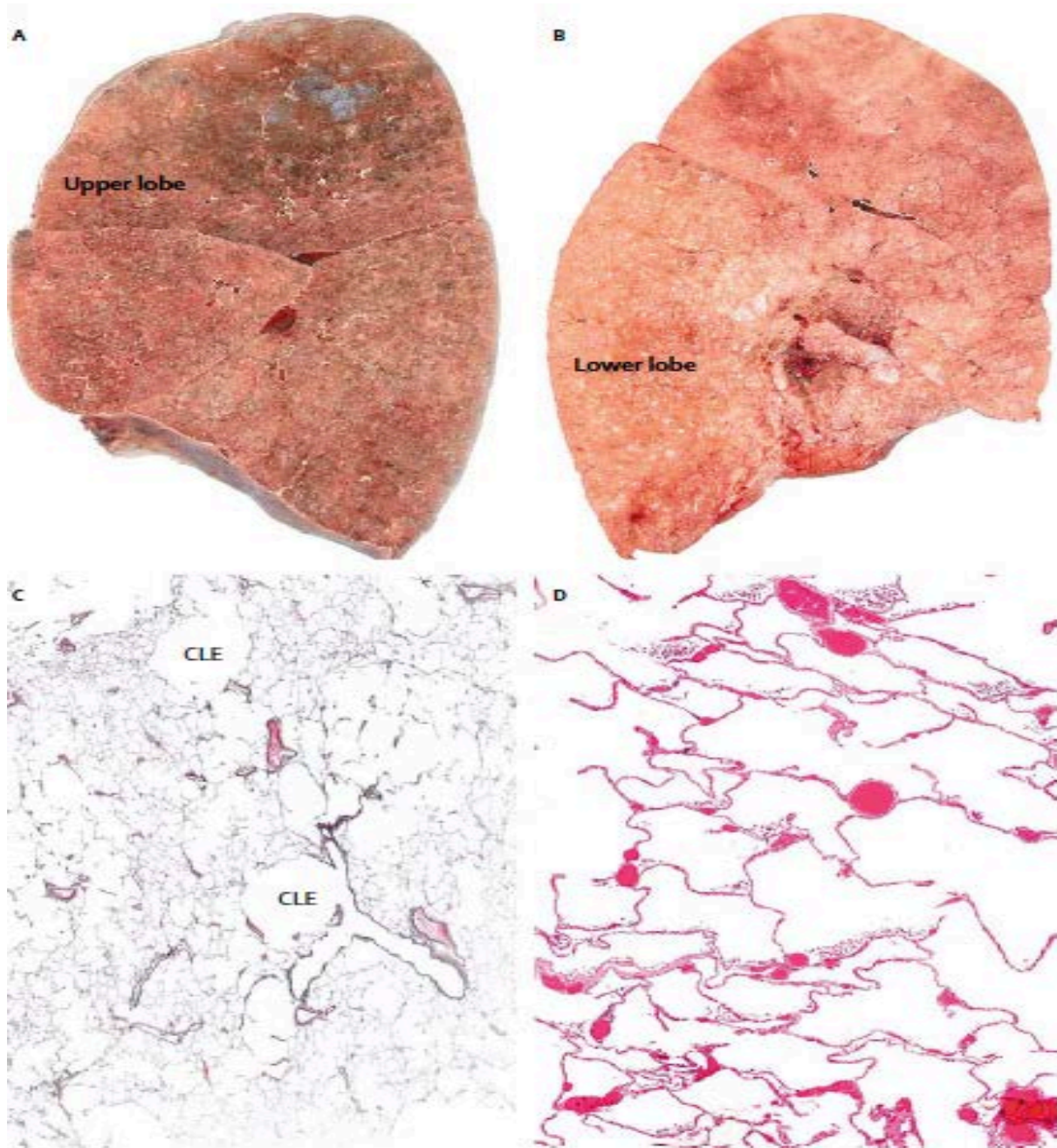


Figure 2-5: Emphysema

(A) Centrilobular lesions have coalesced to produce severe lung destruction in the upper lobe.

(B) More extensive association of the lower lobe by panacinar emphysema.

(C) Low-power photomicrograph of centrilobular emphysema (CLE) that has cracked central portions of several acini of a single lobule.

(D) High power microscopy presenting even obliteration of the lobule in panacinar emphysema.

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2.4 Classification (Physiological – Spirometric)

According to GOLD, diagnosis of COPD requires objective spirometry assessment for the confirmation of definite post-bronchodilator airflow obstruction (GOLD 2015). Spirometry is a reproducible breathing test which is quick, easy, safe and non-invasive for patients. Rather arbitrary spirometry obstructive indices were introduced into clinical medicine many years ago for the definitive diagnosis of air flow obstruction. Special emphasis has been made to the ratio on forced expiratory volume in 1 second (FEV₁) to the forced expiratory vital capacity (FVC), the FER (Hyatt & Black 1973) for proper diagnosis of COPD. According to accepted criteria, FER of <0.7 confirms the presence of airway obstruction, while FEV₁% predicted, classify severity of COPD (mild ≥80%, moderate 50-80%, severe 30-49%, very severe <30%) (Table 2.1). These indices are mainly related to large airways, while some recent attempts to modernize this on the basis of quantitating the characteristic small airway-related scalloping out of the expiratory limb of the maximal flow-volume curve (Johns, D. P., Walters, J. A. & Walters, E. H. 2014) (Figure 2.6), this is not yet widely used. Attempts to use the FEF₂₅₋₇₅%, a computer assisted measurement of maximum forced expiratory flow over the middle half of the FVC (McFadden & Linden 1972), have also not been widely accepted as this measure lack validity because of its dependency on the measurement of FVC and lack of repeatability (Quanjer et al. 2014). Non-spirometric measures of small airway function, e.g. using forced oscillations (FOT) or multi-breath nitrogen washout techniques are currently being evaluated, but will not be as convenient as using small, modern computerized spirometers.

Underuse of spirometry and lack of proper interpretation of spirometric values makes COPD a largely under-diagnosed condition in the primary care setting especially in its early asymptomatic phases (Walters, JA et al. 2008). This is a great lost opportunity to pick up early disease, although in fact significant in pathological terms (Hogg et al. 2004) but before there is major and irreversible clinical deterioration.

| Disease Severity | (FEV ₁ Predicted) | | | |
|---------------------|-------------------------------|---------|---------|--|
| | ERS | ATS | BTS | GOLD |
| Stage 0: at Risk | – | – | – | Normal spirometry; Chronic symptoms (Cough, Sputum Production) |
| Stage 1: Mild | ≥ 70% | ≥50% | >80% | >80% with or without chronic symptoms |
| Stage 2: Moderate | 50% - 60% | 35%-49% | 31%-80% | 50% -79% with or without chronic symptoms |
| Stage 3: Sever | <50% | <35% | ≤30 | 30% - 49% with or without chronic |
| Stage 4: Very Sever | Terminally ill | | | <30% |

FEV₁ – forced expiratory volume in 1 second; ERS-European Respiratory Society; ATS- American Thoracic Society; BTS- British Thoracic Society; Gold- Global Initiative for Chronic Obstructive Lung Disease.

Table 2.1: Stages of COPD Severity.

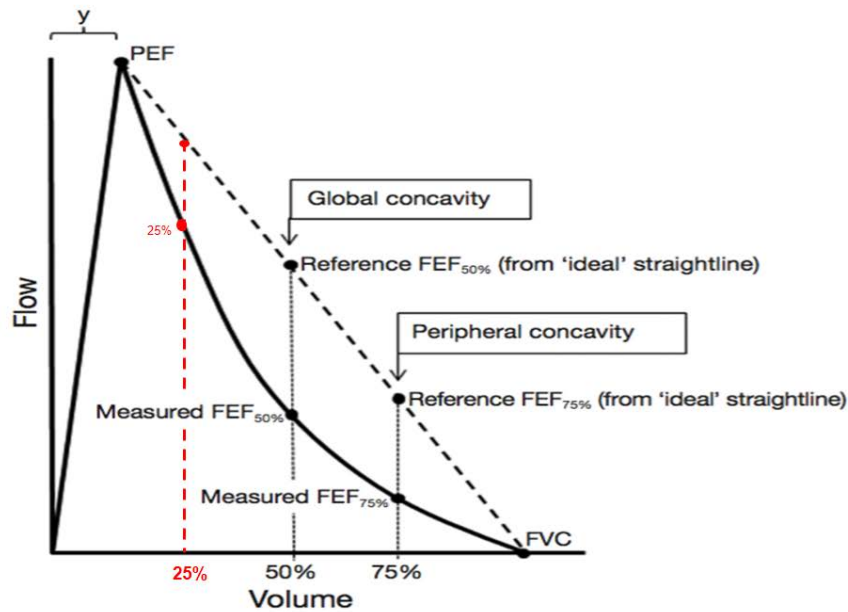


Figure 2-6: Variables used to quantify global and peripheral concavity.

A novel way of interpreting the expiratory limb of the F-V curve. Measured $FEF_{50\%}$ and measured $FEF_{75\%}$ are the forced expired flows when 50% and 75% of the FVC has been expired. Reference $FEF_{50\%}$ and Reference $FEF_{75\%}$ are the reference flows that would be obtained if the flow-volume curve had zero curvature i.e., a linear descending limb (dotted line). The variable, y , is the volume to peak expiratory flow (PEF); a value of 0.6 L can be assumed for this. In this example, global concavity is approximately 50 Units and peripheral concavity is approximately 65 Units. Such an analysis may help in early detection of COPD (by perhaps 25%).

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Another important, widely used and well-evaluated index of lung physiology is DL_{CO} (diffusing capacity for carbon monoxide) which is used mostly in specialist practice. It measures the oxygen-absorbing capacity of the lungs, and basically DL_{CO} is an index of the degree of emphysema in smokers with airflow limitation. This is not useful in early and mild stages of COPD (Bailey 2012).

2.5 Natural history of COPD

A classic, if rather speculative, model of the natural history of COPD in relation to presumably genetic “sensitivity” to smoking was developed almost 40 years ago, though from a series of cross-sectional rather than truly longitudinal data (Fletcher & Peto 1977) (Figure 2.7).

Essentially, the model says that after peak lung function is achieved in one's twenties, a time will come in a decade or so when lung function will start to progressively decline i.e. aging will occur, but in the otherwise healthy this will not have significant impact before death from other natural causes. However, if the subject is a smoker and is (presumably genetically) “sensitive” to the airway and lung damaging effects of smoke then the rate of decline in lung function will be accelerated and COPD and in the worst case premature death will occur. The model can reasonably be modified to apply to most if not all risk factors. The same paper did also mention the possibility that poor lung function going into adulthood in association with even normal rates of lung function decline would also lead to clinical COPD, but this has received rather less attention until quite recently (Perret, J. L. et al. 2016).

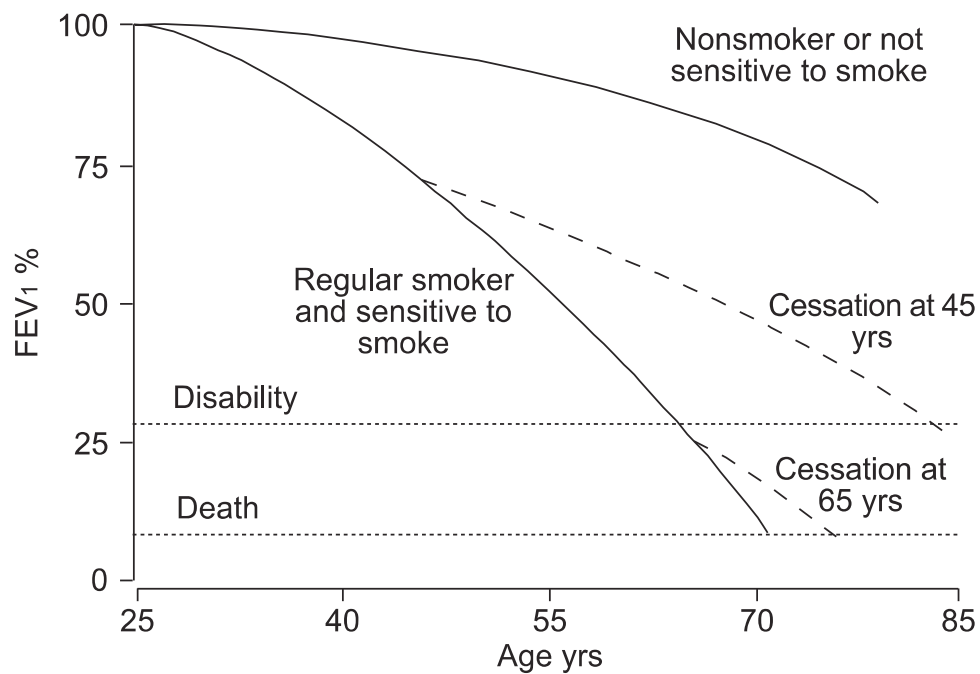


Figure 2-7: Decrease in forced expiratory volume in 1 second (FEV1) according to smoking status and genetic susceptibility.

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2.6 Social and economic impact

2.6.1 Global burden

COPD is the sixth leading cause of morbidity and mortality globally and represents a substantial economic and social burden. As far back as the early 90s, COPD was ranked the sixth leading cause of death had climbed to fourth in 2000, and it is anticipated that it will rank third by 2020 (Lopez et al. 2006). And this does not include the impacts of lung cancers, closely related to COPD (Yang, IA et al. 2011).

Globally, COPD affects approximately 329 million people, which accounts for nearly 5 percent of the total population. According to recent data published in the international collaborative global burden of disease project, COPD is reported to result in increasing mortality rates worldwide, ranging from 2.4 million deaths in 1990, to 2.9 million deaths in 2013 (Global 2013'2015).

In order to directly address local socio-economic impacts of COPD worldwide, the Burden of Obstructive Lung Disease (BOLD) initiative was designed to develop standardized methods for estimating COPD prevalence and resultant economic burden (Buist et al. 2005). In the 2006, BOLD study on 9245 random middle-aged community participants from 12 countries with differing economic development profiles the estimated population prevalence of GOLD stage II or higher COPD was approximately $10.1 \pm 4.8\%$ (SE) overall ($11.8 \pm 7.9\%$ for men and $8.5 \pm 5.8\%$ for women).

The most important factor related to this prevalence in BOLD was pack-years of smoking, along with other associated risk factors whose importance cannot be neglected. These associated factors include mainly third-world usage of biomass fuel for cooking and heating purpose, occupational exposures and multiple respiratory infections (Buist et al. 2007). In South Africa previous TB is another important etiological factor (Jithoo 2006) while in Australia the interaction between the very common active asthma and smoking is very significant (Perret, J. L. et al. 2016). More detailed review of external risk factors is beyond the scope of this Introduction, but is largely summed up in Figure 2.9. Personal “host” factors are dealt with a little later.

In order to understand the overall economic burden of COPD, direct and indirect cost need to be addressed separately and in relevant importance. Direct costs include expenses of admissions to hospitals, home visits, home care, and pharmacological

therapy. The disabling personal and social effects of COPD, as cause of lost working days, are considered as indirect costs (Viegi et al. 2001). In a review of annual direct medical costs of COPD in the USA, in 2005, it was estimated at approximately US\$2700–5900 per patient per year, with US\$6100–6600 for indirect costs. The US National Heart, Lung, and Blood Institute estimated that total national costs 10 years ago (including both direct and indirect costs) of COPD in USA were US\$32.1 billion, with direct costs just over 50% of that (Mannino & Buist 2007). Globally, this cost is anticipated to peak at up to US\$4.8 trillion in 2030, half of which is expected to arise in developing countries (Lomborg 2013), from US\$2.1 trillion in 2010 (Bloom & A.B. 2011).

2.6.2 National burden (Australia)

In Australia, after heart disease, stroke and cancer, COPD is a leading cause of death and socio-economic burden.

According to the Australian Institute of Health and Welfare (AIHW), a decline in death rates for males due to COPD was observed, between 1979 and 2011, while it plateaued up among female from 1979 and 1997 then also starting to decline (Figure 2.8). This difference was due to when smoking became a relevant health risk in the two genders and when quitting began to kick in significantly. In addition to gender, COPD related mortality was reported to be higher among people of indigenous origin compared to non-indigenous Australians (AIHW 2012).

In Australia, COPD was estimated to be the fifth leading cause of death, causing 6,122 deaths in 2011(AIHW 2012). In a recent study from the George Institute, it is reported that not only two-thirds of deaths in current smokers can be directly attributed to smoking, but in addition current smokers are estimated to die an average of over 10 years earlier than non-smokers (Banks et al. 2015).

COPD is costlier per case than other common smoking-related disease such as cardiovascular disease (CVD) and osteoporosis and responsible for substantial burden on the Australia economy (for both direct and indirect cost). COPD is Australia's second leading cause of avoidable hospital admission (Hutchison et al. 2007). In 2008 alone, AU\$98.2 billion were spent on COPD, nationwide, including direct (AU\$8.8 billion) and indirect (AU\$89.4 billion) financial outlays (Economics 2008).

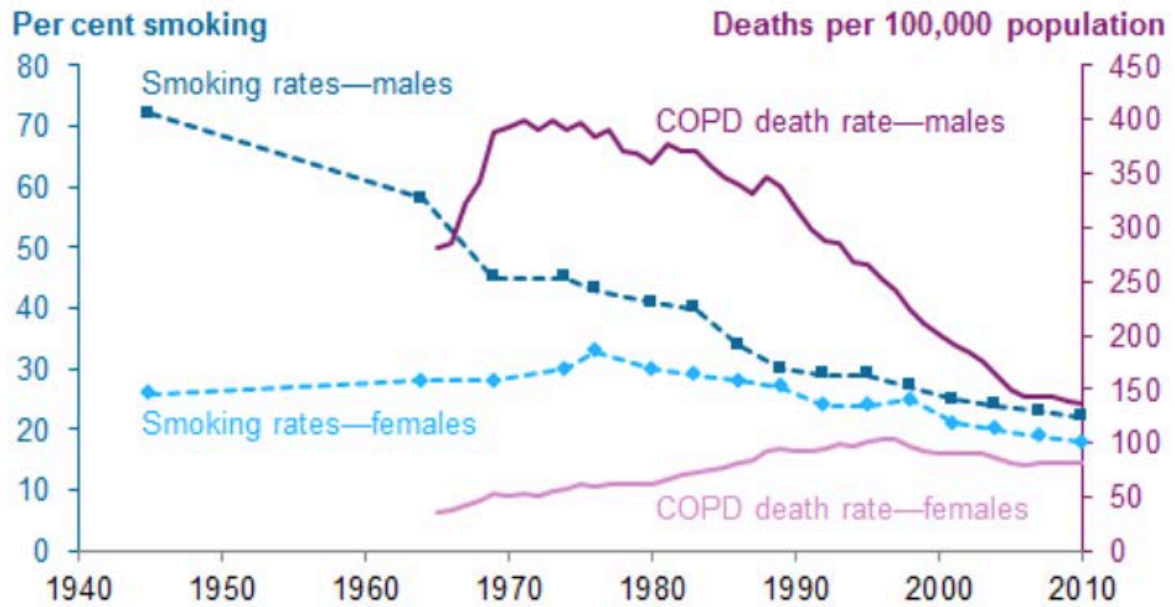


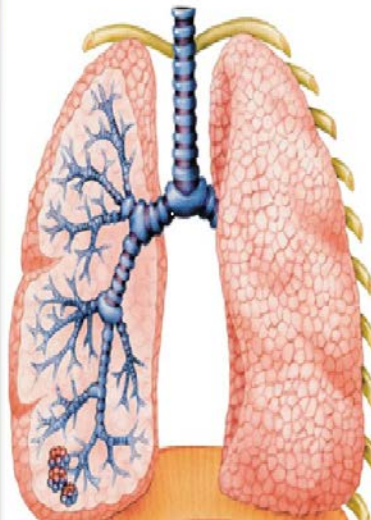
Figure 2-8: COPD death rates in Australia

In relation to smoking rates showing the lag between “peak-smoking” and deaths.

Source: (AIHW 2012).

Exposures

- ✓ Cigarette Smoking
- ✓ Biomass Fuel Exposure (Indoor)
- Asthma
- Air Pollution (Outdoor)
- Occupational Exposure (Dust, organic and inorganic e.g. Byssinosis and coal workers)



Host Factors

- Airway Hyper-reactivity
- α_1 -antitrypsin deficiency
- Age
- Gender
- Lung growth and development
- Oxidative stress

Figure 2-9: Risk Factors for COPD.

2.7 Host factors for COPD

2.7.1 Asthma

Asthma-COPD overlap occurs in patients with features associated with asthma (variably defined as active clinical asthma, bronchial hyper-responsiveness (BHR), allergic bronchial inflammation, reversible airflow obstruction, and increased response to inhaled and systemic corticosteroids) and characteristic of COPD (fixed airways obstruction). A very important study of 228 patients reported that approximately 16% of asthmatic patients not only developed fixed airway obstruction (reduced FEV₁/FEV), but also have typical characteristic features suggestive of COPD (cough, dyspnea, and sputum production) (Vonk et al. 2003). In the Tasmanian Longitudinal Health Study (TAHS), run by our CRE, based on data from 1,389 participants, it has been observed that there is substantial contribution of both current smoking status and clinical asthma, to fixed airflow obstruction in the middle age population, especially in atopic individuals (Perret, Jennifer L et al. 2013)

Barrecheguren and colleagues reported that patients with both characteristics of asthma and COPD has rapid disease progression associated with dyspnea and wheezing and experience more frequent exacerbations and respiratory infections than patients with COPD alone (Barrecheguren, Esquinas & Miravittles 2015).

2.7.2 Genetic Factors

Many smokers are “resistant” to the deleterious effects of smoking and the rate of FEV₁ decline in them is quite similar to nonsmokers (Fletcher & Peto 1977). Therefore, there is likely to be genetic susceptibility for development of COPD, interacting with environmental influences that determine the effect of smoke on the lungs (Hogg et al. 1994).

Several genetic associations have also been found in relation to COPD but detailed analysis of these studies is beyond the scope of this thesis. So I will briefly discuss the apparent most important such factors associated with the development of COPD.

Eriksson and colleagues described the classical genetically determined deficiency of alpha-1 anti-trypsin (anti-protease) and its inability to neutralize neutrophil elastase (protease) leading to early onset of emphysema (Eriksson 1965).

Stoller and colleagues reported that the hereditary deficiency of α 1-antitrypsin (ATT, encoded by SERPINA1), was responsible for just 5% of COPD cases (Stoller & Aboussouan 2005).

After a huge amount of work over subsequent decades and especially more recently as genomic work has become more feasible, many new polymorphisms have been linked to the development of COPD (Table 2.2).

| <i>Gene/Protein</i> | <i>Polymorphism ID</i> | <i>Function</i> |
|-------------------------------|-------------------------------|--|
| MMP9 | CR99492 | Increase promoter activity |
| MMP1 | rs179950 | Increase Transcription |
| GSTP1 | rs947894 | Increase enzyme activity |
| SERPINA3 | rs4934 | Alters signal peptide |
| HMOX1 | CE000297 | Alter transcription |
| TNFα | rs1800629 | Increase TNF alpha level |
| TNFβ | rs1800469 | Increase TGF beta level |
| IL-13 | rs 1800925 | Increase IL13 production |
| SOD3 | CM941295 | Increase enzyme level |
| CHRNA 3/5 | rs8034191 | Hedgehog interacting proteins (HHIP) |
| AGER | rs2070600 | Interact with NFkB |
| HTR4 | rs3995090 | Alter cytokines response |
| THSD4 | rs12899618 | Modulate calcium binding proteins |
| GSTCD | rs10516526 | Alter intra cellular Chloride channels |

Table 2.2: Genetic polymorphisms relevant to COPD (Wood & Stockley 2006).

2.7.3 Age

There has been considerable semantic dispute about ageing and COPD, but it is generally regarded as an independent risk factor for progressive degenerative changes in lung tissues, affecting both structure and function of lungs, and is considered as a predictor for COPD, and certainly as an extra vulnerability to added risk factors (Decramer & Rossi 2001). In a study by Fukuchi and colleagues, COPD is reported to be three-times higher in the elders of >60 years of age than other age groups (Fukuchi et al. 2004). It is also interesting to observe that after ischemic heart disease (IHD) and stroke, COPD is the third leading cause of disability in individuals over 60 years of age (Global 2013'2015). Ageing changes in the respiratory tract have been categorized as consequence of failure to efficiently eliminate reactive oxygen species (ROS), impaired ability to repair of DNA damage, reduction in anti-ageing molecules (histone deacetylases and sirtuins) and/or stimulation of pro-ageing molecules and telomere shortening (Ito & Barnes 2009).

2.7.4 Gender

COPD had been considered as a disease of males because of their earlier take up as a group of smoking decades ago. Women did not take up smoking in large numbers until after World War II. In late 90s, studies showed that females are more susceptible to effects of smoking than males and at given levels of smoking are at increased risk of development of COPD (Prescott et al. 1997). This is presumed to be largely the result of having smaller caliber airways, and it is still unclear whether any hormonal difference puts females at increased risk for COPD development (Barr et al. 2004; Carlson, CL et al. 2001). In addition, Allen Ramey and colleagues reported that in comparison to males, females with COPD also reported more respiratory symptoms, and also more depression and lower health-related quality of life, (Allen-Ramey, Gupta & daCosta DiBonaventura 2012).

2.7.5 Nutrition

A well-balanced diet seems important in relatively protecting against. The factors involved seem to include both types of food and specific nutrient such as vitamin C, D, E and certain minerals. Thus, cross-sectional studies have found in particular that a “western” diet (refined grains, red meats, sugary and fat-laden foods) is associated with an increased risk of COPD in comparison to Mediterranean diet (breads, cereals,

beans, nuts and seeds, fish, poultry and low intake of red meat) (Varraso et al. 2007). Similarly, several studies have demonstrated a specific beneficial effect of fruits and vegetables on lung health (Keranis et al. 2010). Britton and colleagues reported that deficiency of magnesium and omega-3 fatty acids were both associated with lower FEV₁ than those who had sufficient nutrient intake (Britton et al. 1994; Shahar et al. 1994). A recent study by Foong and Zosky reviewed the role of vitamin D deficiency in COPD disease onset, progression and AECOPD, and concluded that vitamin D plays a key role in lung growth (Foong & Zosky 2013).

2.7.6 Socioeconomic status (SES)

Educational attainment and occupation define socioeconomic status (SES), and seemly independently influence both COPD development, (Miravittles et al. 2010; Pena et al. 2000). Multiple factors including differences in COPD-treatment, smoking, unemployment rates, lack of social relations and living alone, all related to low SES and could influence these effects (Lange et al. 2014), which are still ill-understood.

2.8 Airway inflammation in COPD

There has been a strongly held belief over the last two decades that COPD and its progression to airway remodeling is due to “inflammation” (Figure 2.10). Although this may have truth in regards to the airway lumen with innate immunity activated by inhaled smoke (or other “irritants”) or secondary to infection, the evidence for a role of inflammatory cells in airway wall patho-physiology is quite debatable. For the last many years the literature has reported increases in mainly innate inflammatory macrophages and neutrophils, in the airway lumen in COPD, represented by broncho alveolar lavage (BAL) and sputum samples (Pesci, Balbi, et al. 1998; Pesci, Majori, et al. 1998; Rutgers et al. 2000).

However, there are major contradictions for the pictures reported of inflammation in small and large airway wall (Di Stefano, Antonino et al. 1998; Di Stefano, A. et al. 2001; Saetta et al. 1998; Shaykhiev & Crystal 2013). Thus, Lacoste et al observed decrease neutrophil numbers in the airway wall of chronic bronchitis and COPD patients (Lacoste et al. 1993). However, Di Stefano and colleagues observed increased neutrophil counts in the lamina propria of COPD patient when compared to

smokers without airflow obstruction; but there was no other control in this study (Di Stefano, Antonino et al. 1998).

Macrophages are important regulator of chronic inflammation. Two populations of macrophages have been identified in lung, one predominant in the airway wall tissue and the other alveolar macrophages, within the alveoli and airway lumen. Kaku and colleagues reported an overall increase in total numbers of macrophages in the airway lumen of patients with COPD and accepted it as firm evidence for inflammatory disease (Kaku et al. 2014). However, our group has found that the number of macrophage are unchanged in both smoking and COPD group, both in small and large airway, even in healthy non-smoking individuals, the numbers of macrophages in the small airway wall are three fold higher than in large airway (Eapen et al. 2015).

Eosinophilic airway inflammation traditionally has been associated with allergic asthma rather than COPD. An early study by Lacoste et al compared BAL fluid, peripheral blood and biopsy sample eosinophils in asthma with COPD and normal control, and the latter two groups were quite similar (Lacoste et al. 1993). Later studies have variably reported that in COPD, there are increased levels of eosinophils in BAL, induced sputum and the airway wall in COPD (Chung, K. F. & Adcock 2008; Rutgers et al. 2000). This paradox may be related to eosinophilia only being present in an eosinophilic subgroup (30%), which also has excess eosinophils in induced sputum and blood and is the group in COPD which responds best to corticosteroids (Bafadhel et al. 2011; Mori et al. 2015; Singh et al. 2014) It was in line with previous studies which observed that increased eosinophils in BAL and sputum of some COPD patients was related to a better response to corticosteroid therapy (Chanez et al. 1997; Fujimoto et al. 1999; Pizzichini et al. 1998), Reid et al from our group did not find this eosinophil signal in the context of bronchodilator responsiveness in COPD (Reid, D. W. et al. 2008).

Mast cells also may play a vital role in airway innate immune function (John & Abraham 2013) although classically they are described as degranulating, with release of various mediators including histamine, bradykinin and arachidonic-derived lipids, after allergen exposure in sensitized individuals and then causing local vasodilation and smooth muscle contraction. In COPD, data regarding mast cells involvement is sparse. Initially, Saetta et al observed that mast cells number remain unchanged in the

bronchial mucosa of chronic bronchitis subjects (Saetta et al. 1993). Later Grashoff and colleagues reported increased numbers of mast cells in airway epithelium without any change in the rest of the airway wall (Grashoff et al. 1997). Then a study by Di Stefano and colleagues reported normal airway mast cell numbers again in normal lung function smoker and COPD current smokers (Di Stefano, Antonino et al. 1998) while in the large airways Ekberg-Jansson et al observed a significant rise in mast cells in both the epithelium and sub-epithelial airway wall of asymptomatic smokers (Ekberg-Jansson et al. 2005). Soltani et al in our group recently observed increases in the number of mast cells in the epithelium and LP of COPD current and ex-smokers in large airway biopsies, but this increase was especially around blood vessels (Soltani, Ewe, et al. 2012). In the small airways, Andersson and colleagues reported a significant decrease in number of mucosal mast cells in the sub epithelium of small airways across COPD GOLD stage I-IV subjects (Andersson, CK et al. 2010), but we have been unable to replicate that (unpublished data). It is difficult to know what to make of these conflicting and contradictory data on airway mast cells, but it does emphasise the need for well-phenotyped subjects and adequate comprehensive control tissues.

The role of **dendritic cells** in COPD is not very much clearer. Dendritic cells are monocyte derived cells which play an important role in initiating adaptive immune response in airways as elsewhere, by recognition, uptake, processing and presentation of antigens to lymphocytes, i.e. as antigen presenting cells. Some studies have reported a decrease in mature dendritic cells (CD83+) in small airway and alveoli of COPD subjects, both current and ex-smokers (Tsoumakidou et al. 2009). Later another group reported that cigarette smoke induced an increase in number these CD83+ cells in lung tissue in COPD. (Vassallo et al. 2010). In our group Sohal et al observed very few CD11c+ve dendritic cells, closely related to basal epithelial cells and the Rbm of large airways in COPD patients (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2011). In agreement with this, another recent study observed no significant change in the number of dendritic cells in small airway wall in COPD (Utokaparch et al. 2014).

The role of **CD8 T lymphocytes** (cytotoxic or suppressor adaptive cells) is perhaps better established in the pathology of COPD and particularly in disease progression, though again there are major contradictions in the literature. Shaughnessy et al

reported that the numbers of CD8+ T cells were increased in the LP of bronchial biopsies in “chronic bronchitis” patients and inversely correlated with their airway obstruction (FEV1) (O'Shaughnessy et al. 1997). However, Di Stefano et al later reported that not only were lymphocytes decreased in COPD but this decrease progressively paralleled worsening disease severity (Di Stefano, A. et al. 2001). Eapen et al from our group recently, also reported a decrease in both CD8+ and CD4+ T lymphocytes in the LP of the large airway wall with increasing disease severity, but with a ratio skewed abnormally toward CD8+ T lymphocytes, in comparison to healthy non-smoker controls (Eapen et al. 2015). They also observed an increase in CD8+ T lymphocytes in the lamina propria of the small airway wall which was in contrast to previous finding by Utokeaparch and colleague who didn't find any significant change in CD8+ T lymphocytes in small airways of COPD subjects versus their normal control (Utokeaparch et al. 2014). Overall, on balance the evidence seems to suggest at least a bias towards a CD8+ lymphocyte balance in COPD, but the evidence is rather thin.

In short, current evidence on “inflammation” in COPD is inconsistent and contradictory across different type of cells in the airway wall component. At the very least the dogma around a significant role for chronic inflammation in the pathogenesis of airway remodeling as the core abnormality in COPD is highly questionable.

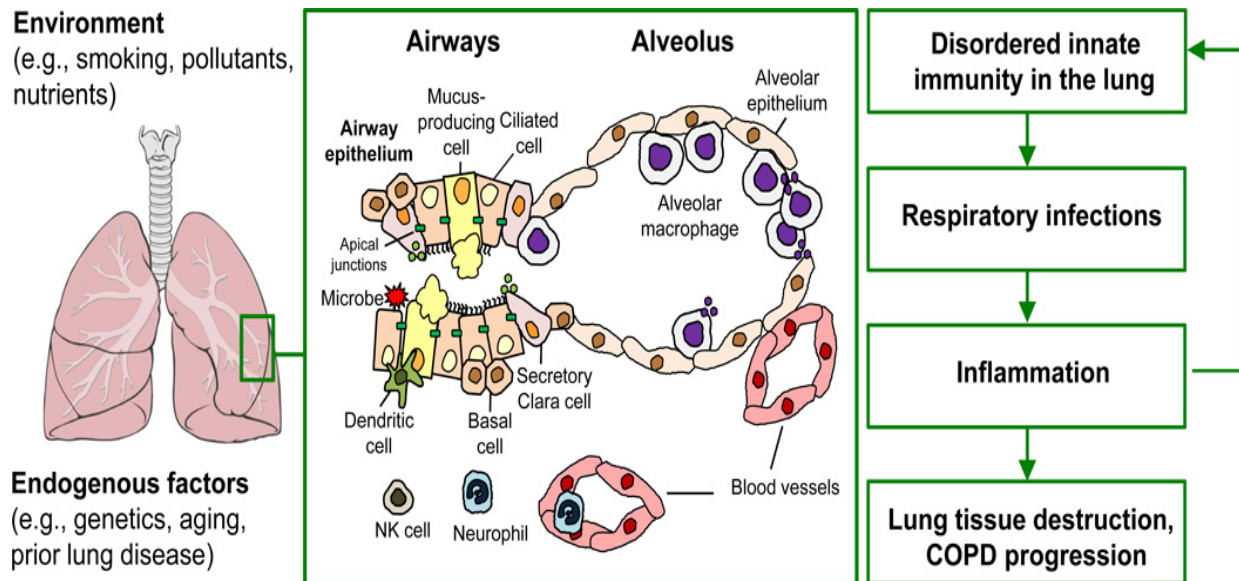


Figure 2-10: Current dogma on the role of disordered innate immunity in COPD pathogenesis.

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2.9 Airway Remodeling in COPD

Airway remodeling is the vital core feature in COPD, some of which is the cause and mechanism for progression and severity of airflow obstruction. I have already introduced this concept but will now deal with it in greater detail, given its centrality to the aims of my work.

Current data with now strong supporting evidence is that remodeling is not a consequence of airway inflammation, but it precedes or may even cause inflammation (Hirota & Martin 2013; Postma et al. 2014).

Airway remodeling has been defined rather clumsily as: “structural alteration in the airway wall that occur as consequence of multiple factor associated with disease pathogenesis during the course of chronic airway disease” (Boulet & Sterk 2007). It is characterized by the changes in tissue, cellular, and molecular composition, affecting epithelium, the sub-epithelial part of the mucosa including airway smooth muscle, blood vessels, and extracellular matrix. Classically, the pathologic characteristics of remodeling in the large airways in COPD has been limited to epithelial squamous metaplasia and goblet cell hyperplasia, sub-mucosal gland hyperplasia, and smooth muscle hypertrophy (Bergeron & Boulet 2006; Shapiro, SD & Ingenito 2005; Walters, E. H. et al. 2008), plus infiltration of various inflammatory cells in the LP and sub-mucosal glands (O'Shaughnessy et al. 1997) (Figure 2.11). I will now briefly update here this picture has developed to, taking each tissue component in turn.

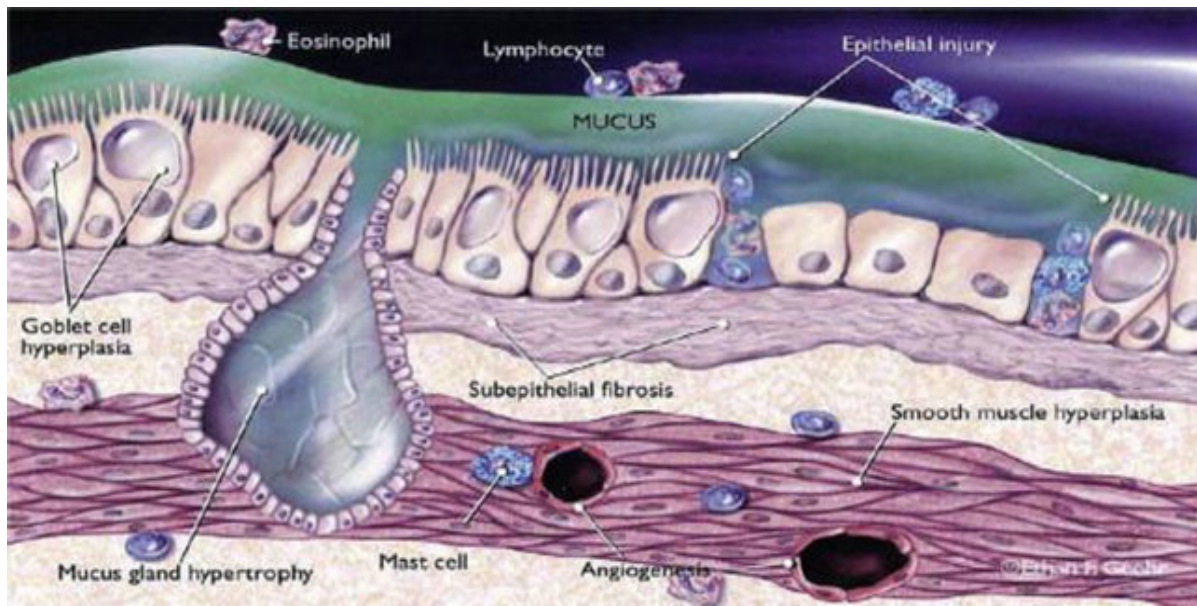


Figure 2-11: Airway Remodelling.

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2.9.1 Epithelium

The pseudostratified respiratory epithelium lining the trachea and bronchi (proximal airways), is made up of three major cell types on the basis of their structure and function: 1) Apical ciliated cells, 2) non-ciliated secretory cells, and 3) basal (stem) cells. Epithelium is of course the layer to come into contact with inhaled irritants like cigarette smoke, and a crucial physical, chemical and immunological protective barrier to its impacts (Araya et al. 2007; Knight & Holgate 2003; Thorley & Tetley 2007).

Although denuded epithelium has been observed in young smokers (Cosio et al. 1980) this was probably something of an artifact, but major pathological events in COPD associated remodeling, after the pioneering work of Reid, Dunnill and others over 50 years ago, have been confirmed to be thickened epithelium with squamous metaplasia, goblet cell hyperplasia and loss of apical cells cilia (Hirota & Martin 2013; Kim, Victor, Rogers & Criner 2008)

Squamous metaplasia of bronchial epithelium is a pre-neoplastic change that occurs as a result of cigarette smoke induced toxic injury (Barsky et al. 1998; Peters et al. 1993; Rigden et al. 2016) (Figure 2.12). It is a complex multistep process which may eventually lead to neoplastic transformation, i.e. bronchial carcinoma. Squamous metaplasia doesn't progress to a neoplasia in all smokers, particularly if low grade and can regress back to normal epithelium after smoking cessation (Araya et al. 2007; Breuer et al. 2005; Jeremy George et al. 2007; Lapperre et al. 2007; Wistuba et al. 1999).

Goblet cell hyperplasia is common in smokers/COPD, regardless of the presence or absence of symptomatic chronic bronchitis (Hogg et al. 2004; Innes et al. 2006; Reid, L 1954). It has been shown that subjects with more airflow obstruction have a greater burden of mucus in the airways. In addition, a bronchoscopic study in smokers with and without airflow obstruction demonstrated more goblet cell hyperplasia in the large airways and in COPD (Saetta, M. et al. 2000) (Figure 2.12).

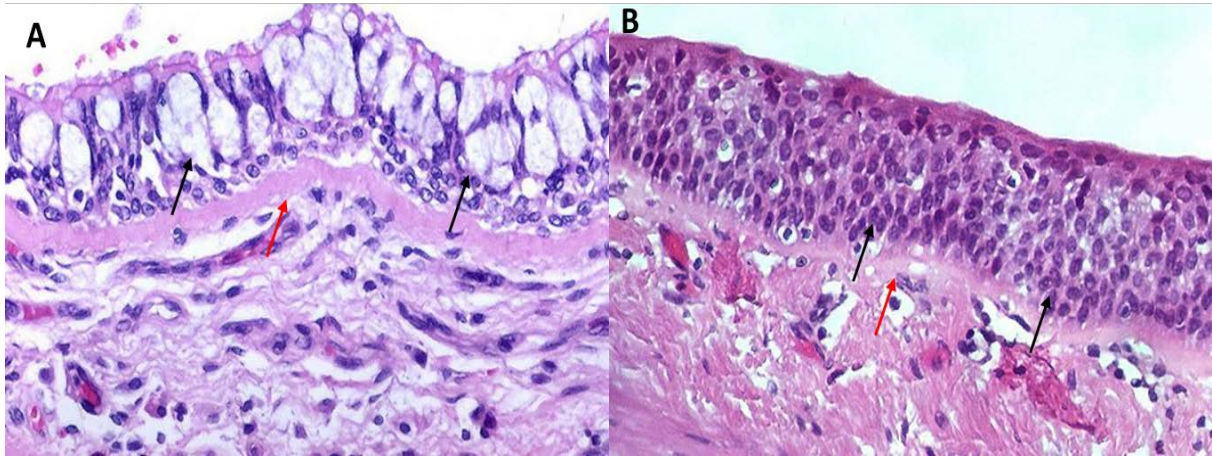


Figure 2-12: Airway epithelium in COPD.

A) Goblet cells hyperplasia (Black arrows). B) Squamous metaplasia (Black arrows)
And Thick Rbm (Red arrow)

Cigarette smoke exposure is likely to activate multiple mechanisms underlying these changes, but oxidative pathways are likely to be especially important, leading to the secretion of cytokines and epithelial derived growth factors by epithelial cells, airway smooth muscle cells, T lymphocytes, mast cells, eosinophils and macrophages. (Saha et al. 2009). The epidermal growth factor receptor (EGFR) may play an especially vital role. Dysregulated and persistent EGFR activation has been observed in association with metaplastic and/or hyperplastic airway epithelium in both asthma and COPD patients (Holgate 2011; Woodruff 2011). Recently, Ganesan and colleagues also reported that aberrant activation of EGFR in airway epithelial cells is associated with increased production of the neutrophil chemokine IL-8, via a PI3K/Akt-mediated pathway (Ganesan et al. 2013).

Matrix metalloproteinases (MMPs) are also thought to play a major role in aberrant epithelial repair of smoke induce “damage” to airway epithelium in COPD lungs, with elevated expression of MMP1, 2, 8, 12, 14, and especially MMP9 reported (Atkinson & Senior 2003; Elkington & Friedland 2006). Two major components of cigarette smoke, Acrolein and lipopolysaccharide (LPS) enhanced the activity and expression of MMP9 in COPD airway epithelial cells in vitro (Jermini, Weber & Grandjean 1976; Wang, Y et al. 2009). Notably, persistent exposure to cigarette smoke can increase EGFR activity via MMP9.

I will be dealing with evidence for EMT in smoker’s epithelium elsewhere.

2.9.2 Reticular basement membrane (Rbm)

The human airway epithelium is separated from the underlying lamina propria and its mesenchymal constituents by a true basement membrane (BM). Immediately below this is the reticular BM (Rbm) or lamina reticularis.

Collagen IV (for strength), laminin (adhesive protein), and proteoglycans (structural protein) are the three major components of the true BM. Beneath this the Rbm is normally composed of collagen I, III, V, and tenascin. Both layers are strongly connected by strands of collagen VII. Laminin and tenascin have binding sites for other matrix proteins, as well as for mesenchymal and epithelial cells. These matrix proteins along with proteoglycans, form a complex and stabilized network that regulates multiple cell processes such a cell-migration and cell-adhesion (Liesker et al. 2009).

Rbm thickness has been widely reported in asthma, with a homogeneous, hyaline and thickened appearance (Ward, C et al. 2002). But data regarding Rbm appearance in COPD are sparse and contradictory. For instance, Vachier and colleague reported a normal Rbm (Vachier et al. 2004) in COPD, while Bourdin et al observed the Rbm to be thickened (Bourdin et al. 2007) here. A series of studies by our group in smokers/COPD, reported that the Rbm was not only abnormally thick in COPD, but also fragmented, hyper-cellular and hyper-vascular (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010) (Figure 2.13). In addition, Rbm fragmentation was positively correlated with individual smoking histories in COPD subjects (Soltani, A. et al. 2010). These changes have been dealt with in greater detail earlier. Our evidence on the Rbm has recently been replicated by Gohy and colleagues (Gohy, S. T. et al. 2015).

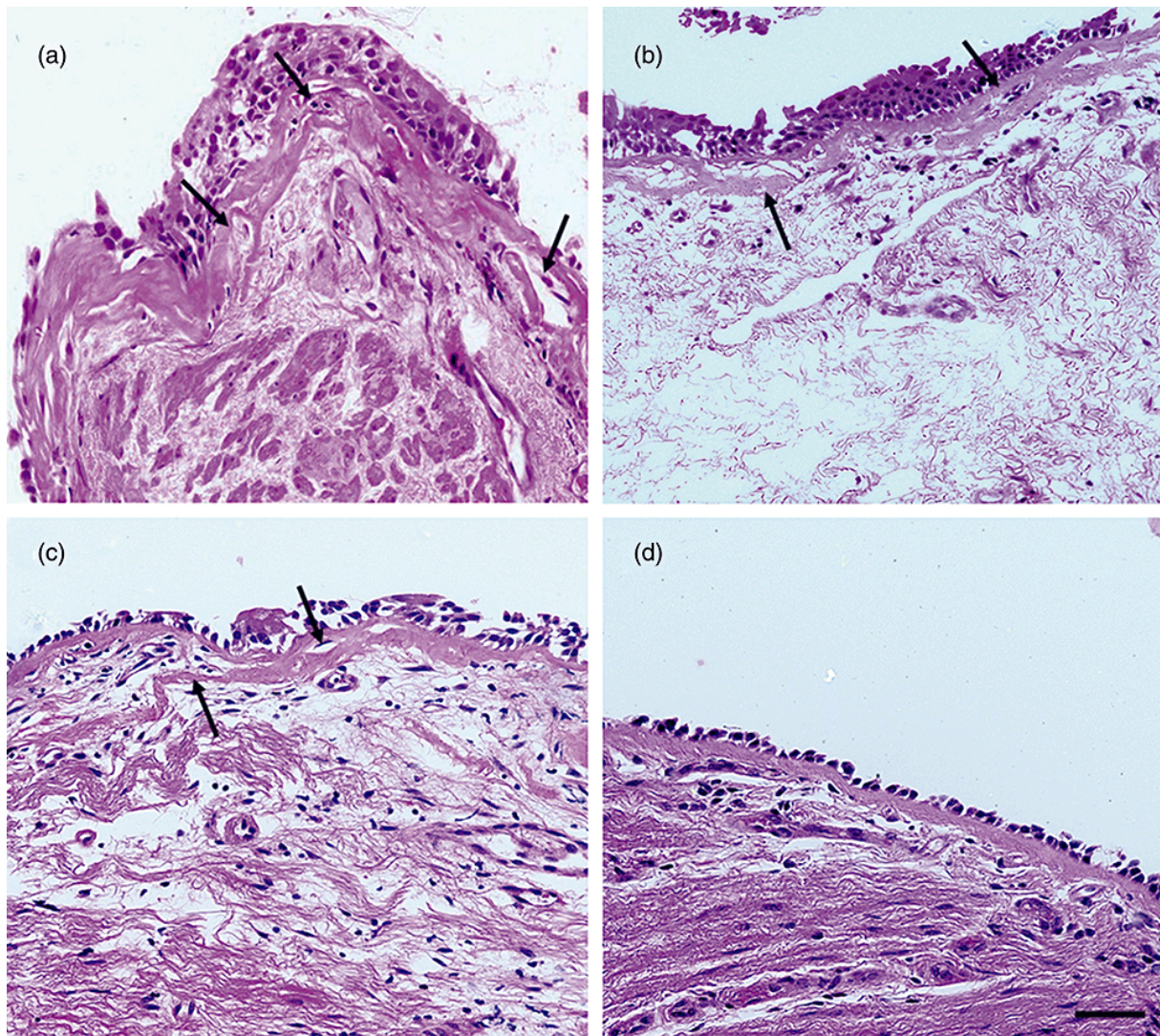


Figure 2-13: Rbm Thickness and fragmentation with cells in it.

Bronchial biopsy section from (a) a current smoker with COPD, (b) an ex-smoker with COPD, (c) a smoker with normal lung function and (d) a normal control subject

Reproduce with permission from Sohal, S. S., Reid, Soltani, Ward, Weston & Muller (2010), Copyright Wiley publisher.

2.9.3 Airway smooth muscle

Airway smooth muscle has been regarded mainly as a contractile organelle, but more recently it has been found that it can itself become inflammatory and modulate neighbouring cells and matrix, including in COPD. Thus, airway smooth muscle cells are able to proliferate and secrete growth factors, cytokines, prostanoid, and extracellular matrix proteins and play a key role in airway remodelling (Jeffery, P. K. 2001).

In COPD, small airways smooth muscle contractile hyper-responsiveness has been reported and due to calcium-dependent and calcium-independent mechanisms (Su 2014). Many inflammatory mediators, growth factors, and reactive oxygen species (ROS), contribute to the alterations of airway smooth muscle functionality in COPD (Hardaker et al. 2004; Hay, D 1999; Hirst, Twort & Lee 2000; Karpova et al. 1997; Michaeloudes et al. 2015; Wiegman et al. 2015).

Gosens and colleagues reported increased expression of β -catenin (intra cellular signal transducer) in human airway smooth muscle cells in COPD (Gosens et al. 2010). Later Baarsma and colleagues observed that β -catenin signalling was activated in response to TGF β 1 in airway smooth muscle cells, and was required for production and regulation of ECM protein in COPD (Baarsma, Hoeke A et al. 2011), so making β -catenin dependent gene transcription, a potential target as a therapeutic intervention in airway remodelling in COPD. Details regarding β -catenin, are given later in this chapter.

ROS induced oxidative stress has been implicated in airway smooth muscle pathology in COPD. Thus, NADPH oxidase (NOX4, which produces hydrogen peroxide) protein expression was elevated in airway smooth muscle of COPD and controls with significant smoking histories and was completely abrogated by NOX4 inhibition in those subjects with or without COPD. So NOX4 may play a role in hydrogen peroxide-induced ROS generation in COPD (Hollins et al. 2016). They also reported that the intensity of this expression in airway smooth muscle from COPD subjects was enhanced in the presence of TNF α , which is increased in COPD (Chiang, Chuang & Liu 2014).

A role for vitamin D in COPD has not been fully established completely. However, vitamin D treatment inhibited secretion of inflammatory mediators in stimulated airway

smooth muscle cells, and also inhibited proliferation of stimulated smooth muscle cells in COPD (Banerjee & Panettieri 2012).

Most studies on smooth muscle mass have been in small airways rather than large, although various studies have observed that thickness of the large airway wall was associated with reduced FER (FEV1/FVC) (Chung, Kian Fan 2005; Jeffery, Peter K 2004). Presumably smooth muscle changes are part of this, but more data are required.

2.9.4 Submucosal glands and mucus hypersecretion

Some but not all patients with COPD show characteristics of airway mucus hypersecretion (sputum production and increased luminal mucus), due to goblet cell hyperplasia and submucosal gland hypertrophy, plus decreased clearance of mucus because of cilia dysfunction (Kim, V. & Criner 2013). Such airway hypersecretion has pathophysiological and clinical significance in COPD, causing patients to be more prone to respiratory tract infections. In comparison to asthma, it is interesting to note that mucus in COPD patients differs in a sense that: 1) it is less viscous and marked plasma exudation is absent and 2) the ratio of mucin MUC5AC:MUC5B may be reduced (Rogers 2000).

Histologically submucosal glands are composed of two secretory units: serous and mucus. Reid et al first described submucosal gland hypertrophy in 1954 (Reid, L 1954). In 1960 Glynn et al reported that in chronic bronchitis this mucus and serous ratio of submucosal glands is disturbed, with decrease in serous and increase in mucous gland units in comparison to normal (Glynn & Michaels 1960). Normally, there is a wide range of antibacterial substances secreted by the serous unit, including lysozymes, IgA and lactoferrin, so relative loss of these add potential for chronic bacterial colonisation and overt infection in the airways in COPD (Jeffery, P. K. 2001).

Mechanistically, mucus hypersecretion in COPD is a consequence of cigarette smoke exposure itself (Deshmukh et al. 2005), plus acute and chronic bacterial and viral infection (Holtzman et al. 2005) and/or inflammatory cell activation of mucin gene transcription (Burgel & Nadel 2004). Gland neutrophilia through neutrophil elastase release is involved in overproduction and hypersecretion of mucus, as might be EGFR activation (Takeyama et al. 2001). As mentioned, all of this is compounded by a difficulty in clearing the secretions because of poor ciliary function, and also distal

airway occlusion, and an ineffective cough as a result of reduced peak expiratory flow (Verra et al. 1995).

Smokers with and without COPD have reduced chloride conductance in the lower airway which has some resonance with cystic fibrosis, and this ion transport abnormality is associated with dyspnoea and chronic bronchitis (Dransfield et al. 2013). Upregulation of the fibroblast growth factor receptor (FGFR1) and TGF β as well as a higher frequency of the TNF- α polymorphism have also been implicated in the pathogenesis of chronic bronchitis (Guddo et al. 2006; Vignola et al. 1997) (Figure 2.14). EGFR also been reported to involve in goblet cell hypertrophy and hypersecretion of mucus in smoker COPD patients. Takeyama et al reported cigarette-smoke induced EGFR activation increased mucin MUC5AC synthesis in the airway epithelial cells (Takeyama et al. 2001). Further details are given in the specific EGFR section.

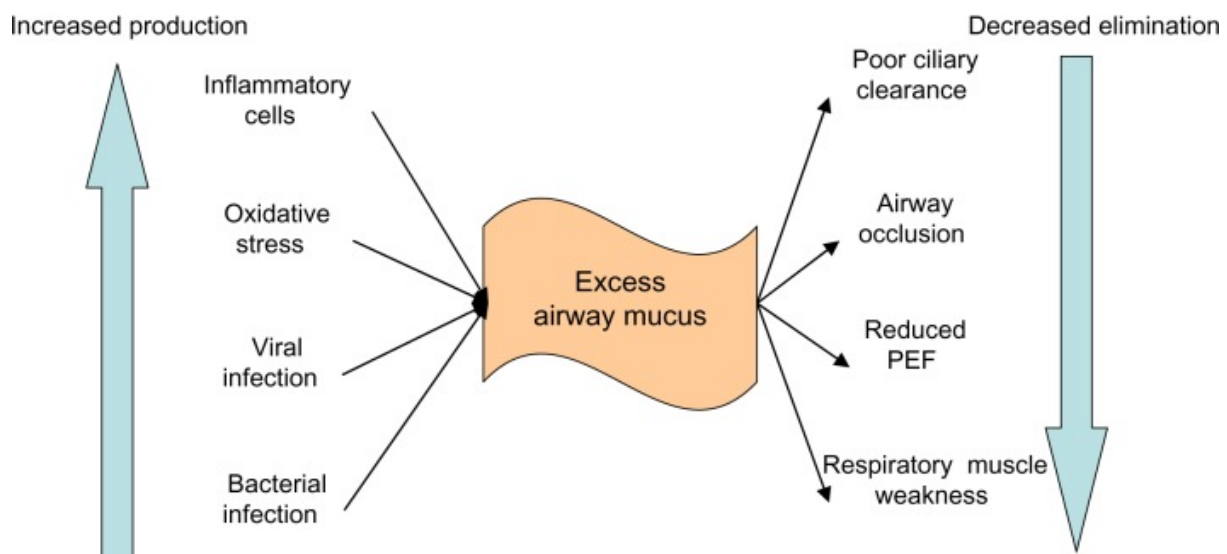


Figure 2-14: Causes of excessive mucus production in COPD.

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2.9.5 Extracellular Matrix (ECM)

The tissue ECM is an amorphous material, made up of fluids and a combination of proteoglycans and glycosaminoglycans. Important matrix proteins include collagen particularly I, III and IV, decorin, laminin, tenascin, versican, biglycan, perlecan, hyaluronan and lumican. These ECM proteins are reported to be involved in number of physiological functions, including: structural integrity; cellular migration; assembly and aggregation of structural proteins; elaboration and storage of growth factors and cytokines; fluid balance and osmotic activity. Change in ECM matrix proteins may lead to change in these respective functions. However, data regarding matrix changes and their pathogenic role in COPD are relatively sparse, and there are discrepancies among the studies that are available.

Recent research has emphasized on expressions of major ECM components such as collagen, elastin, and proteoglycans, where were observed to be altered in COPD, compared to healthy smokers or healthy nonsmoker controls, summarized in Table 2.3. In particular, I would point out the study by Van Straaten et al indicating that in comparison to non-smokers or patients with mild emphysema, the airways of smokers with severe emphysema had decrease immune-histochemical expression of the proteoglycans biglycan and decorin in peribronchial tissues, but without changes in collagen, fibronectin or laminin (Van Straaten, J et al. 1999). Biglycan and decorin play an important role in cross-linking collagen fibrils, therefore their loss would imply dysfunctional tissue integrity. However, several other studies reported that in central airways of mild to moderate COPD, collagen Types 1 and 3 are increased in the basement membranes, lamina propria, and adventitia, and this change negatively correlated with lung function parameters i.e. more such “scarring” greater was the airway obstruction (Harju et al. 2010; Kranenburg et al. 2006; Liesker et al. 2009).

In addition to airways, alveolar components of ECM are likely to be disturbed in COPD. Thus, the volume fraction of elastic fibres was decreased in alveolar tissue and bronchioles in COPD patients (Black et al. 2008; Eurlings, Irene MJ et al. 2014; Hogg, J. C. & Timens, W. 2009; Merrilees et al. 2008). These findings are concordant with the findings of Zhang et al that fibroblasts, isolated from distal lung of COPD patients,

have impaired capacity to produce elastin in response to TGF- β 1 (Zhang et al. 2012).

| ECM protein | Mild/moderate COPD Small airways | Mild/moderate COPD Parenchyma | Severe COPD Small airways | Severe COPD Parenchyma |
|-----------------------|--|---|--|---|
| | References | References | References | References |
| Elastin | ↓ (Eurlings, I. M. et al. 2014) | ↓ (Eurlings, I. M. et al. 2014) ↓ (Merrilees, M. J. et al. 2008) ↔ (Annoni et al. 2012) | ↓ (Eurlings, I. M. et al. 2014) | ↓ (Eurlings, I. M. et al. 2014) ↑ (Deslee et al. 2009) ↑ (Vlahovic et al. 1999) |
| Biglycan | ↔ (Annoni et al. 2012) | ↔ (Annoni et al. 2012) | ↓ (Van Straaten, J et al. 1999) | ↓ (Van Straaten, J et al. 1999) |
| Decorin | ↔ (Annoni et al. 2012) | ↔ (Annoni et al. 2012) | ↓ (Zandvoort et al. 2006) ↓ (Van Straaten, J et al. 1999) | ↓ (Zandvoort et al. 2006) ↓ (Van Straaten, J et al. 1999) |
| Versican | ↔ (Annoni et al. 2012) | ↑ (Merrilees, M. J. et al. 2008) ↓ (Annoni et al. 2012) | ↑ (Hallgren et al. 2010) | ↑ (Hallgren et al. 2010) |
| Total Collagen | ↑ (Eurlings, I. M. et al. 2014) | ↑ (Eurlings, I. M. et al. 2014) | ↑ (Eurlings, I. M. et al. 2014) | ↑ (Eurlings, I. M. et al. 2014) |
| Collagen I | ↓ (Annoni et al. 2012) ↑ (Harju, T. et al. 2010) ↑ (Kranenburg, A. R. et al. 2006) | ↓ (Annoni et al. 2012) | ↓ (Harju, T. et al. 2010) | |
| Collagen III | ↔ (Annoni et al. 2012) ↓ (Harju, T. et al. 2010) | ↔ (Annoni et al. 2012) | ↓ (Harju, T. et al. 2010) | |
| Collagen IV | ↔ (Annoni et al. 2012) | ↔ (Annoni et al. 2012) | | |
| Tenascin | ↑ (Annoni et al. 2012) ↑ (Lofdahl et al. 2011) ↑ (Liesker, J. J. et al. 2009) | ↔ (Annoni et al. 2012) | | |
| Fibronectin | ↑ (Annoni et al. 2012) | ↔ (Annoni et al. 2012) | ↓ (Gosselink et al. 2010) | |
| Hyaluronan | ↑ (Eurlings, I. M. et al. 2014) ↑ (Dentener et al. 2005) | ↑ (Eurlings, I. M. et al. 2014) | ↑ (Eurlings, I. M. et al. 2014) | ↑ (Eurlings, I. M. et al. 2014) |
| Laminin | ↑ (Kranenburg, A. R. et al. 2006) ↔ (Liesker, J. J. et al. 2009) ↔ (Van Straaten, J et al. 1999) | | | |

Table 2.3: Extracellular matrix changes in airways and parenchyma of patients with COPD.

2.9.6 Cartilage

The airway cartilage may act as a significant brake against excessive airway narrowing with airway smooth muscle contraction, but only limited data are available regarding cartilaginous changes in COPD. In comparison to asthma, where the amount of airway cartilage has been observed to be either increased or unchanged (James 1997), reduced amounts of cartilage have been reported in COPD. (Tandon & Campbell 1969). Similarly, cartilaginous degeneration and perichondrial fibrosis, have also been observed in “chronic bronchitis and emphysema” (Haraguchi, Shimura & Shirato 1999) contributing to expiratory collapsibility of the airway wall. Inoue and colleagues reported clinically relevant chondromalacia causing intense dynamic compression of the airways during expiration (Inoue, M et al. 2009). We do not know how such changes may be generally relevant to more common COPD pathology.

2.9.7 Airway vascularity and Angiogenesis

Angiogenesis means new vessel formation that involves the formation of thin-walled endothelium-lined structures with muscular smooth muscle wall and pericytes, and plays an essential role as “repair mechanism” of damaged tissues. Normal lung vessel homeostasis maintains specific density of vessels per unit area of the lungs; which is observed to be disturbed in chronic lung diseases (Voelkel, Douglas & Nicolls 2007). Lungs of course have double vascularization: the bronchial circulation deriving from the thoracic aorta (via intercostal or mammary arteries), and the pulmonary circulation from the right ventricle forming the parenchyma air/blood interface (Kayser et al. 1997). My interest is in the bronchial vessels.

There are multiple angiogenic factors identified as, playing a pivotal role in tissue associated angiogenesis, including the fibroblast growth factor (FGF) family, VEGF, angiogenin, GM-CSF, TGF α and TGF β , TNF α , PDGF, hepatocyte growth factor (HGF), chemokines, angiopoietin and epidermal growth factor-like domain 7 (EGFL7) (Nichol & Stuhlmann 2012; Postma, D. S. & Timens, W. 2006; Puxeddu et al. 2005).

Although angiogenesis is a complex process requiring tightly coordinated balancing between a variety of these regulator, VEGF has been thought to be one of the most important in chronic inflammatory environments (Knox, Stocks & Sutcliffe 2005).

VEGF is secreted by multiple cells including endothelial cells, macrophages, stromal cells and malignant cells, but the main target is the endothelium (Potente, Gerhardt & Carmeliet 2011) (Figure 2.15). There are five forms (A, B, C, D, E) but the most important in angiogenesis is VEGF-A. (Senger et al. 1983), which has six isoforms: VEGF₁₂₁, VEGF₁₄₅, VEGF₁₆₅, VEGF₁₈₃, VEGF₁₈₉, VEGF₂₀₆; all VEGF isoforms are able to bind the receptor tyrosine kinase VEGFR-2. The VEGFR-1 receptor seem to be an inactive decoy, and part of homeostatic mechanisms (Feltis et al. 2006). In addition to directly stimulating endothelial cells to proliferate, a fundamental role of VEGF in angiogenesis is to stimulate the secretion and activation of proteolytic enzymes (matrix metalloproteases, plasminogen activator), to degrade the ECM to allow proliferating endothelial cells to migrate and organize into tubular structures (Ferrara 2009).

In context of COPD, angiogenesis has been observed not only associated with progression of airway remodeling but potentially able to control and orchestrate it (Reid, D. W. et al. 2008; Walters, E. H. et al. 2008). Some studies have suggested that vascular remodeling in COPD might only start at moderate degrees of disease severity (Kessler et al. 2001).

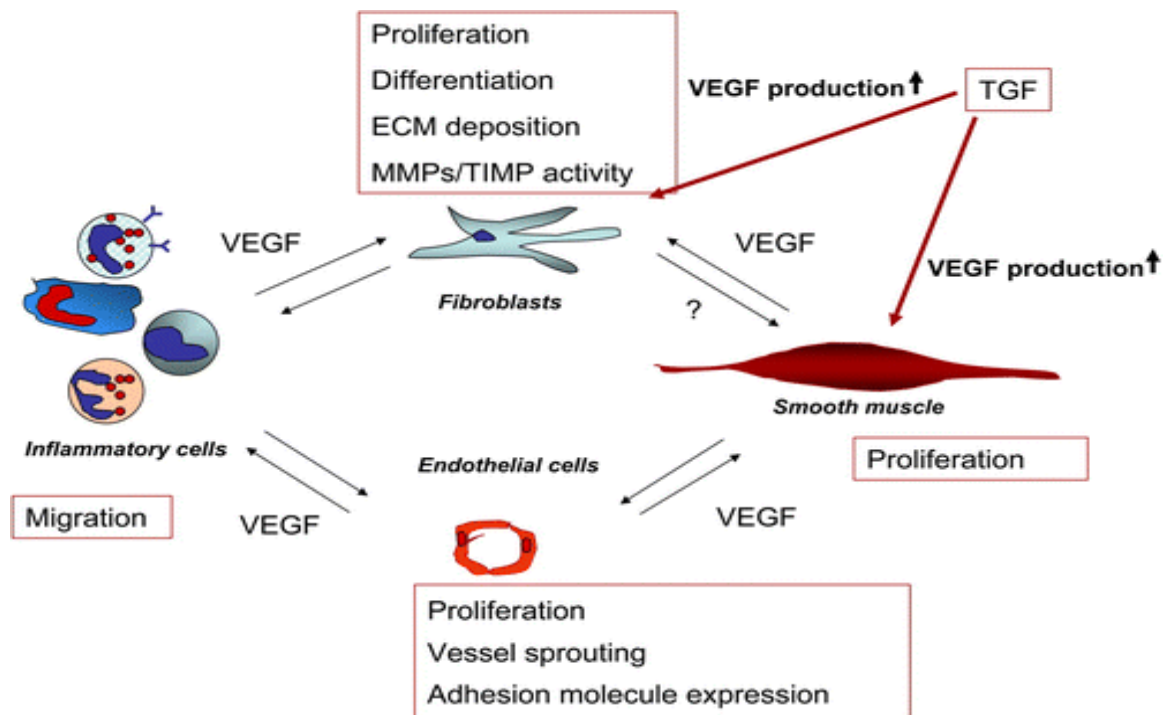


Figure 2-15: Schematic representation of the main roles of VEGF.

ECM=extracellular matrix; TGF=transforming growth factor; MMP=matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase.

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A study by Kranenburg and colleagues reported that COPD was associated with an increased expression of VEGF in the epithelium of bronchi, bronchioles, and alveoli, in bronchiolar macrophages, as well as airway smooth muscle and vascular smooth muscle cells both in bronchiolar and alveolar regions (Kranenburg et al. 2005). They also postulated that VEGF and its VEGFR-1 and VEGFR-2 receptors may be involved in epithelial and endothelial cell repair in response to cigarette smoke induced injury and might be associated with airway remodeling in COPD. Increased expression of VEGF was found to be associated with integrin $\alpha v \beta 3$ (Santulli et al. 2011).

Some studies have used bronchial biopsies for studying airway vessels in volunteers. Calabrese et al reported an increased number of vessels and VEGF positive cells in the airway LP of smokers in comparison to healthy non-smokers (Calabrese et al. 2006), but Zanini et al could not find a difference in vessel numbers between normal controls and COPD ex-smokers although vascular area, VEGF and TGF β expressing cells were greater in COPD (Zanini et al. 2009).

More recently, our group has undertaken the most comprehensive studies so far on airway vessels in smokers and COPD, with for the first time optimal controls. Using BB from normal controls, smokers with normal lung function, currently smoking and ex-smoker COPD. Soltani et al observed large airway Rbm hyper-vascularity but LP hypo-vascularity in current smokers irrespective of their lung function, with surprisingly COPD ex-smokers being not different from healthy non-smoking controls. In fact, hypo-vascularity of the whole bronchial arterial system in the COPD airways were first described 60 years ago (Cudkowicz & Armstrong 1953). In contrast to the LP hypo-vascularity, VEGF positive vessels and number of vessels were observed to increase in the Rbm of current smokers with normal lung function and COPD groups (Soltani, A. et al. 2010), with a remarkable positive correlation between LP hypo-vascularity and air trapping due to small airway dysfunction. In contrast, there was a positive correlation between FEV1% predicted and VEGF expressing vessels in the Rbm in current smokers with COPD (Soltani, A. et al. 2010). Thus, these pathological changes do seem to be physiologically relevant. One of my first priorities was to repeat some of this work in small airways.

2.10 Airway remodelling and lung function

As described previously, the major site of obstruction in COPD is in the small airways (Hogg et al. 2004). In these airways remodelling (and possibly chronic inflammation) are strongly associated with airflow obstruction (Bosken et al. 1990; Hogg et al. 2004). Moreover, increased structural abnormalities especially in airways epithelium, lamina propria and smooth muscle showed a strong association with disease progression in COPD (Hogg et al. 2004). In general, such conclusions about the importance of airway remodelling in COPD have been relatively ignored; indeed, only recently has research interest in potential underlying mechanisms of these key changes become evident. Our group has been at the forefront of this new move, and my work I hope will now also have made a significant contribution.

2.11 Epithelial mesenchymal transition (EMT)

EMT represents conversion of an epithelial cell into an elongated cell with mesenchymal phenotype, which can occur both in physiologic and pathologic processes (Hay, ED 1995). Being influenced by several intra-cellular biochemical mechanisms, EMT is mainly associated with loss of cell polarity, inter-cell membrane adhesions dissolution, cytokeratin filaments disintegration and potential migration of the newly formed cells that now have a mesenchymal phenotype (Kalluri, R. & Neilson, E. G. 2003; Kalluri, R. & Weinberg 2009). Besides increase in migratory capacity, cell resistance to apoptosis also increases, accompanied by excessive production of both matrix metalloproteinases (MMPs) and extracellular matrix components (Kalluri, R. & Neilson, E. G. 2003; Kalluri, R. & Weinberg 2009; Zeisberg, EM et al. 2007) (Figure 2.16).

The first description of EMT was in 1982 by Elizabeth Hay who initially termed the process “epithelial mesenchymal transformation” and then in 1995 showed its involvement in embryogenesis and foetal organ development (Guarino, Tosoni & Nebuloni 2009; Hay, ED 1982, 1995). The term “transformation” has been replaced by “transition” on the basis of its potential induction and potential reversibility (Kalluri, R. & Neilson, E. G. 2003). Thus, along with EMT, a reverse process called mesenchymal-epithelial transition (MET), has been also been reported, not only in embryogenesis (Kalluri, R. & Weinberg 2009) but also associated with stromal tumor development and metastasis (Liu, S, Cui, J, et al. 2014; Liu, S, Liao, G, et al. 2014).

Histologically, epithelial and mesenchymal cells create multi cellular structures which are quite different in their visual appearance and morphology (Shook & Keller 2003). A typical epithelium is composed of sheet of cells, usually one cell thick, with uniformly arranged individual epithelial cells, with intercellular adhesion and tight junctions holding them together as a single unit and inhibiting individual cell movement away from the monolayer sitting on a common collagenous basement membrane. Epithelial polarity means that the basal and apical surfaces of these cells are not only visually different but also adhere to different substrates with different functional capacity. However, typical mesenchymal cells do not exhibit tight inter-cellular adhesion between them and they form irregular structures which are not of uniform composition. For this reason, much less direct interaction and association exists between

mesenchymal cells in comparison to their epithelial counterparts, which augment their migratory capacity. Moreover, migration pattern of mesenchymal cells is mechanistically different from epithelial cells. Epithelial cells move in the form of sheet, whereas mesenchymal cells are considerably more dynamic and move individually (Table 2.4).

Epithelial-Mesenchymal Transition

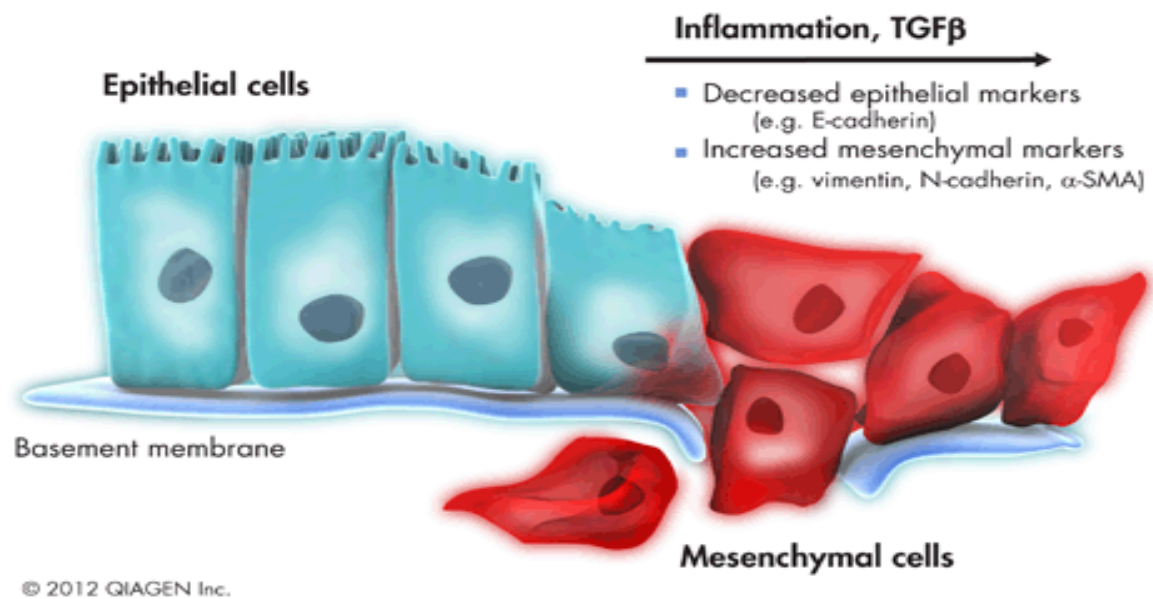


Figure 2-16: Epithelial mesenchymal transition (EMT).

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| | Epithelial | Mesenchymal |
|---------------------------|---|--|
| Morphology | Round cluster, cobblestone-like Uniform monolayer Contact inhibition | Elongated, spindle-shaped Scattered Reduced cell—cell contact |
| Polarity | Apical—basolateral | Leading edge |
| Intercellular junction | Tight Adherens Desmosome Gap | Focal adhesions Transient gap |
| Cytoskeletal organisation | Circumferential belt | Filapodial stress fibres |
| Motility | Limited, sheet migration | Enhanced, individual cell migration |
| Invasiveness | Restricted | Elevated |
| Molecular markers | E-cadherin ZO-1 Claudin-1 Occludin Desmoplakin Cytokeratin-8, -18, -19 | N-cadherin Vimentin Fibronectin α smooth muscle actin Snail Slug |

Table 2.4: Epithelial and mesenchymal cell characteristics.

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2.11.1 EMT classification

Based on the physio-pathogenic nature, EMT is classified into three main subtypes: (Figure 2.17).

Type 1 EMT, involved in embryogenesis (organ development), mainly designed to generate primary mesenchyme (Kalluri, R. & Weinberg 2009; Zeisberg & Neilson 2009). EMT-Type 1 is implicated in the formation of the mesoderm from the epithelium and in the generation of neural crest cells. This transition process is potentially reversible with re-transformation of resultant primary mesenchyme into secondary epithelium. This process of MET is required for completion of the cellular differentiation process and for orientation of the three dimensional structure of organs. These cycles of both EMT and MET have been reported to be involved in the development of the heart, liver and islets of Langerhans in pancreas (Johansson & Grapin-Botton 2002; Nakajima, Y et al. 2000; Nawshad et al. 2005; Tanimizu & Miyajima 2007).

Type 2 EMT is involved in non-neoplastic fibroblast recruitment in chronic inflammation-related wound healing, tissue repair and tissue regeneration (Bataille et al. 2008; Kalluri, R. & Neilson, E. G. 2003; Kalluri, R. & Weinberg 2009; Zeisberg, EM et al. 2007; Zeisberg & Neilson 2009). However, these processes of repair can become excessive and then EMT is associated with tissue fibrosis. In this fibrotic EMT-related process, accumulated fibroblasts secrete collagen fibres, leading to organ dysfunction. For instance, in the lung, Kim KK et al have demonstrated that alveolar epithelial cells in patient with idiopathic pulmonary fibrosis (IPF) undergoes EMT thus contributing to the perenchymatous fibrosis and lung restrictive physiological change (Kim, KK et al. 2006).

Type 3 EMT represents cytoskeleton disorders that result from dissolution of cell-cell adhesion, cell scattering and finally loss of apical-basal cell polarity in malignant epithelial tumors (Kalluri, R. & Weinberg 2009; Liu, F et al. 2014; Whipple et al. 2010; Zhang, JX et al. 2014). During metastasis, the mobile nature of these cells allows them to migrate through the ECM/tissue and reach the blood circulation to find new metastatic sites in target organs. At the same time metastatic cells also express mesenchymal markers such as α -SMA (Smooth muscle actin), vimentin, fibroblast specific protein-1 (FSP1; also known as S100A4) or desmin (Yang, J & Weinberg 2008). These markers are particularly expressed in the cells localised at the invasion

front of the tumour and are associated with the detachment of these cells from the ECM (Brabletz, Hlubek, et al. 2005; Fidler & Poste 2008; Thiery, J. P. 2002). It is also interesting to note that EMT is not only associated with metastatic potential of malignant cells but also associated with development of a pre-malignant stroma especially when associated with angiogenesis (Kalluri, R. & Weinberg 2009; Soltani, Sohal, et al. 2012).

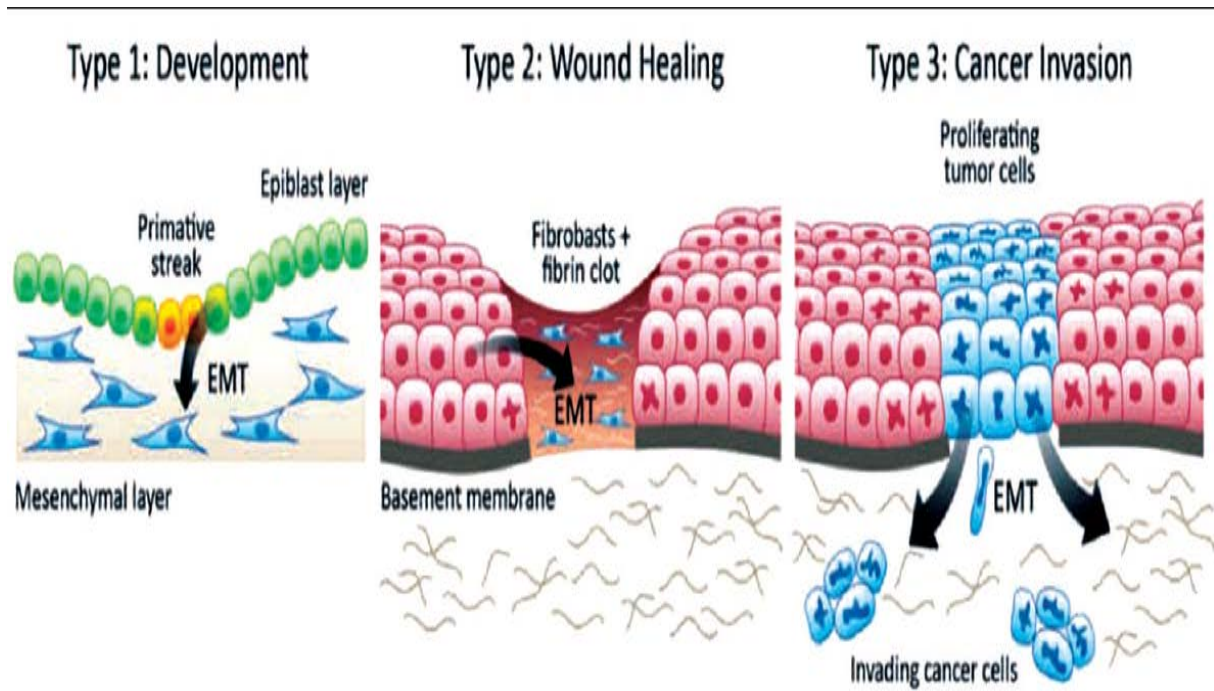


Figure 2-17: Types of EMT.

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2.11.2 Aetiology and Mechanism of EMT

EMT, both in disease or foetal development, is under control of specific pathways, external drivers activate cell surface receptor and in turn specific pathways, transcription factors and ultimately specific genes. However, at a more macro-level it also depends on: 1) the tissue microenvironment 2) the epithelial and stromal cell context and 3) exogenous changes in host immunity, for initiation of EMT the programme i.e. development of the mesenchymal phenotype within formerly classic epithelial cells; increased cell motility; and, in many cases, an ability to degrade and replace ECM proteins to augment the invasive behaviour of these transitioned cells. Moreover, cells that have undergone EMT exhibit increased resistance to senescence and apoptosis as well (Kim, Y-S et al. 2014).

Identification of EMT activity requires a selection of specific markers. The loss of the epithelial phenotype is characterised by a decrease in the expression of the cytoskeleton (CK), other epithelial proteins including junctional proteins (zonula occludens-1 and E-cadherin), and also decreased expression of the surface protein MUC1 (Mucin 1, cell surface associated) (Figure 2.18). Along with this loss, the acquisition of mesenchymal markers is more difficult to explain on the basis of their expression (Lee, J. M. et al. 2006). The main issue with typical mesenchymal marker is that these are confounded by presence of proteins such as vimentin which are present in non-mesenchyme cells such as leukocytes and endothelial cells as well (Okada et al. 2000) and FSP1 (S100A4) is expressed by some inflammatory cells, endothelial cells and smooth muscle cells (Gibbs et al. 1995; Inoue, T et al. 2005; Le Hir et al. 2005; Strutz et al. 1995). A diversity of mesenchymal markers is needed for specificity of EMT detection in tissue. But this is aided by the positioning of the markers e.g. by epithelial basal cells, and structural changes to the reticular basement membrane such as fragmentation due to migrating cell enzymic digestion, hypercellularity to cell-digestion due to the presence of these phenotypically mesenchymal cells (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010).

The local cell environment and generation of strategic growth factors is highly specific for each tissues, where it plays a vital role in activating the signalling pathways leading to EMT (Finkel 2003; Radisky 2005). Thus factors that induce EMT under some

circumstances can have quite different effects in others. For instance, hyperactive Raf/MAPK pathway is required for TGF β -induced EMT (explained later), tumorigenesis, and metastasis. In contrast, activation of the PI3K component of this pathway is required for protection of cells from TGF β -induced apoptosis, allowing scattering but not EMT and causing tumour development but not metastasis (Janda et al. 2002). These strategic growth factors include epidermal growth factor (EGF), connective tissue growth factor (CTGF), insulin-like growth factor 2 (IGF), fibroblast growth factor 2 (FGF-2), interleukin 1(IL) and hepatocyte growth factor (HGF) (Gavert & Ben-Ze'ev 2008). For instance, in kidney cells, FGF2 induces EMT by helping to stimulate the secretion of degradation enzymes like MMP2 and MMP9, resulting in the characteristic fragmentation of basement membrane (Strutz et al. 2002) Similarly, IGF2 induces E-cadherin and β -catenin dissociation from the cell surface membrane which, as I will discuss, can be a major potentiator of EMT (Morali et al. 2001). HGF is mainly associated with embryogenesis (EMT Type 1), especially in cardiac bud formation (Mizuno et al. 1998), but HGF can act with the transcription factor Snail, to reduce the E-cadherin (CHD1) expression, thus destabilising membrane adhesion complexes to again release β -catenin and so ultimately activating pro-mesenchymal genes in the epithelial cell (Grotegut et al. 2006).

Some mechanisms of EMT are summarized in Table 2.5. There are several other putative smoking related mechanisms for induction of EMT which are introduced later in the section of this chapter on “Smoking and EMT”.

| Category | Components | Reference |
|-----------------------|---|---|
| Growth Factors | IGF-1 HGF EGF CTGF | (Liao et al. 2014) (Farrell et al. 2014) (Buonato, Lan & Lazzara 2015) (Zhu et al. 2015) |
| Transcription factors | Smad2/3 B-catenin Snail Twist | (Câmara & Jarai 2010) (Jiang, YG et al. 2007) (Wang, Y et al. 2013) (De Craene & Berx 2013) |
| Pathways | TGFβ-Smad pathway Wnt-Fzd pathway PI3K/AKT pathway MAPK pathway Hedgehog pathway uPAR pathway Notch pathway | (Soltani, Sohal, et al. 2012) (Lamouille, Xu & Derynck 2014) (Lamouille et al. 2012) (Huang, M et al. 2015) (Xu, X et al. 2014) (Wang, Q et al. 2013) (Niessen et al. 2008) |
| Receptors | TGFβ AhR A7-nAChR EGFR | (Vignais & Fafet 2005) (Ono et al. 2013) (Zhang, C et al. 2016) (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010) |

Table 2.5: Mechanisms of EMT.

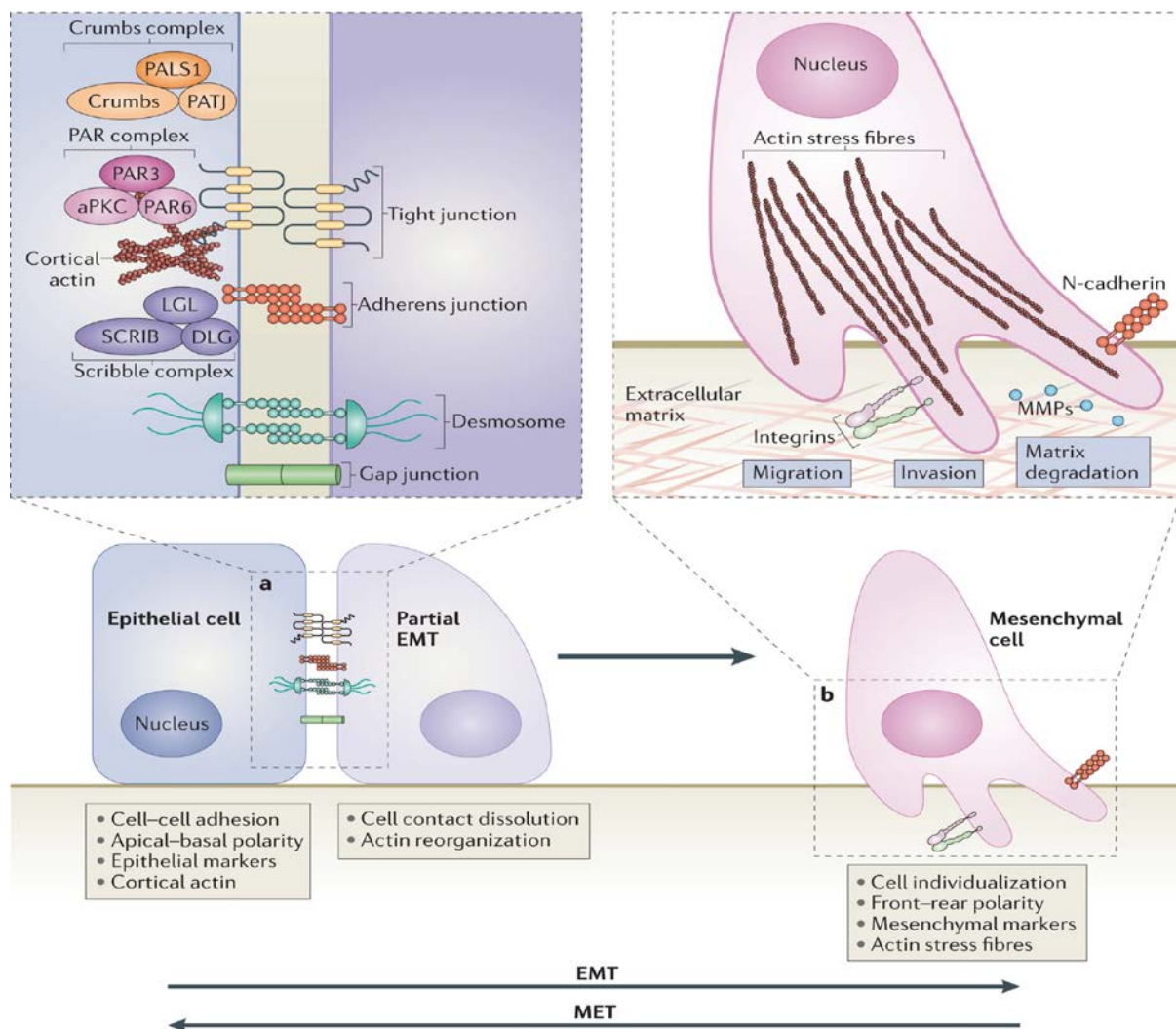


Figure 2-18: Cellular events during EMT and MET.

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2.11.3 EMT Signalling Pathways

2.11.3.A TGF- β (1) signalling pathway(s)

The Transforming Growth Factor Beta (TGF-beta) superfamily consists of the TGF- β 1 proteins, Bone Morphogenetic Proteins (BMPs), Activins, Inhibins Growth Differentiation Factors (GDFs), Glial-derived Neurotrophic Factors (GDNFs), and Müllerian Inhibiting Substance (MIS).

TGF- β 1 is a multifunction cytokine having key roles during development, inflammation, repair, proliferation and cell differentiation. During development TGF- β is involved in stimulating EMT Type 1, especially well-described in cardiac valve and neural crest formation and during palate fusion (Nawshad, LaGamba & Hay 2004; Nawshad et al. 2007; Sridurongrit et al. 2008). On the other hand, in pathological events like lung cancer, TGF- β 1 acts as a double-edged sword, as in the early stages it acts as a tumour suppressor, but in more advanced stages TGF- β 1 favours EMT Type 3, so contributing to the invasion and metastases.

TGF- β 1 is also considered as a 'master switch' for fibrogenesis in disease processes of many organs, including the lung (Takizawa et al. 2001; Willis & Borok 2007; Zhang, M et al. 2009). In vitro studies using pulmonary epithelial cells obtained from healthy donors, exposure to TGF- β 1 induced a transition to mesenchymal phenotype in these cells (Borthwick et al. 2009; Câmara & Jarai 2010; Doerner & Zuraw 2009; Gardner et al. 2012; Hackett, TL et al. 2009; Heijink et al. 2010; Zhang, M et al. 2009).

2.11.3.A.i Smad-dependent TGF- β 1 pathway

Smads are transcription factors (Roberts, de Caestecker & Lechleider 2000) including regulated Smads (R-Smads), grouped into receptor-activated Smads 1, 2, 3, 5, 8 and 9; a common co-mediator Smad4 and inhibitory Smads 6 and 7 (Liu, F, Poupponnot & Massagué 1997; Nakao et al. 1997). TGF- β 1 signalling via Smads is the best characterized pathway that is known to induce EMT, and this is true also of other members of TGF- β superfamily of ligands (TGF- β 1, TGF- β 2, and TGF- β 3 and Activins). The details are quite complex, presumably because close control is needed for such a potentially important process.

With TGF- β agonist binding to its Type I and Type II receptors, a hetero-tetrameric complex activates its tyrosine-kinase activity. This then activates the “canonical” TGF- β signalling cascade involving recruitment and activation phosphorylation of the R-Smads 2 and 3 (Massague 2000; Schiller, M., Javelaud, D. & Mauviel, A. 2004). In order to enable nuclear translocation of these pSmads, a pSmad2/3 dimer forms a complex with Smad 4 to form a Smad 2/3/4 complex which is translocate to inside of the nucleus where it binds to gene regulatory elements to transcriptionally regulate key genes associated with EMT (Derynck & Zhang 2003; Fuxe, Vincent & Garcia de Herreros 2010) (Figure 2.19). Nuclear transport of pSmad 2/3/4 complex depends upon certain nucleoporins (proteins which are the constituent building blocks of the nuclear pore complex), including Sec13, Nup75, Nup93 and Nup205 (Chen, X & Xu 2011). There are also cytoplasmic inhibitory Smads (6 & 7) involves in control: they are activated phosphorylated by the pro-inflammatory cytokines TNF- α and IFN- γ which can themselves be transcriptionally regulated by TGF- β , as well as by Epidermal Growth Factor (EGF) and ultraviolet radiation (Yan, X, Liu & Chen 2009).

Over-expression of Smad 7 blocks Smad-3-dependant EMT by repressing both the transcription of EMT associated transcription factors ZEB1 (zinc finger E-box binding homeobox 1) and Snail1. This effect has been shown in both in vivo and in vitro (Zavadil & Bottinger 2005). The activated R-Smad complex is capable of forming further complex with Snail1 to suppress the expression of genes encoding E-cadherin and Zona occludin-1 which then liberates β -catenin (Cano et al. 2000; Thuault et al. 2006; Vincent, T. et al. 2009). Similarly, in our group, Soltani et al observation in a preliminary study that TGF β 1 was indeed likely to be functionally active through pSmad 2/3 expression in COPD airways (Soltani, Sohal, et al. 2012).

2.11.3.A.ii Smad-independent TGF- β 1 pathway

Signalling through TGF- β receptor complexes can also involve a number of Smad-independent pathways, includes activation of p38, mitogen activated protein kinase (MAPK), Rho GTPases and phosphatidylinositol 3-kinase(PI3K)/Akt pathways (Ellenrieder et al. 2001; Willis & Borok 2007; Xu, J, Lamouille & Derynck 2009). The diversity of functions associated with each pathway is quite substantial. The inhibition of Rho-A by TGF- β facilitated the destabilization of tight junctions while MAPKs was

shown to play a key role in cell membrane adherens junction disassembly, release of β -catenin and induction of cell motility following TGF- β stimulation (Zavadil, J. et al. 2001). The relative importance of the canonical versus non-canonical pathways of TGF- β -induced EMT activity in particular situations such as lung diseases is still unknown, and needs further research studies; the fact that a pathway can be induced under model conditions does not necessarily mean that it is important in human disease.

2.11.3.A.iii TGF- β 1 as a therapeutic target

TGF- β 1 or its downstream pathways might represent potential therapeutic option for the treatment of pulmonary disease either by administration of neutralizing antibodies, use of antisense nucleotides or administration of a TGF- β receptor kinase inhibitor. Recent studies have shown variable success of Pirfenidone (an anti-TGF β agent) for treatment of IPF attenuates TGF- β production and action (Fernandez & Eickelberg 2012) and has a significant if not huge clinical effect. SB431542 which inhibits TGF- β receptor I and R-Smad activation, suppressed experimental bleomycin induced pulmonary fibrosis (Higashiyama et al. 2007; Koh et al. 2015). Finally, Horan et al observed an effect on bleomycin-induced pulmonary fibrosis in mice of a monoclonal antibody against the integrin α v β 6, a key modulator of TGF- β (Horan et al. 2008).

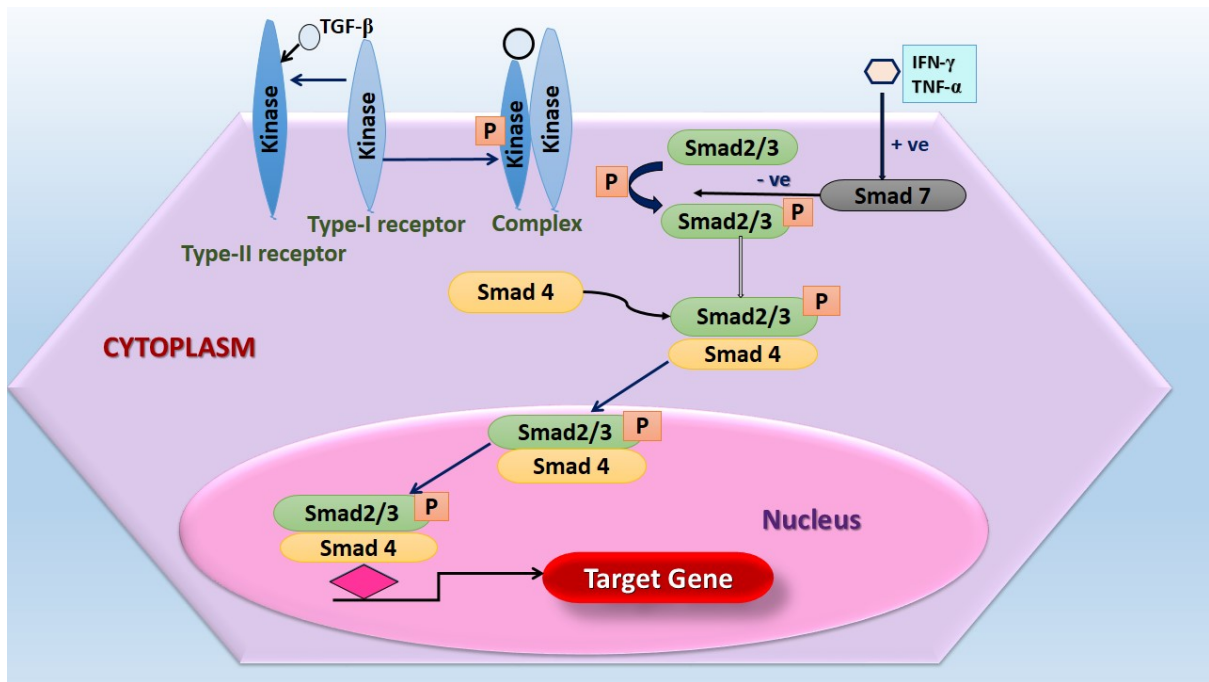


Figure 2-19: TGFβ-Smad dependant pathway.

2.11.3.B Wnt- β catenin signalling pathway

Wnt (wingless tail), is an EMT-driving agonist that mostly exerts its action via the transcription factor β -catenin, having been originally identified as playing a role in carcinogenesis. However, this system also seems to be involved in mechanisms of lung remodelling, and indeed in a variety of other pathological conditions such as IPF, rheumatoid arthritis and type 2 diabetes mellitus (Bordonaro 2009; Eger, A. et al. 2004; Pongracz & Stockley 2006; Sen et al. 2000; Shin et al. 2010; Zavadil, J. et al. 2001). The balance of β -catenin effects (see below) is regulated by how much of it is sequestered in the cell membrane tight junction apparatus, how much is free in the cytoplasm, and how much is sequestered by cytosolic control mechanisms.

Wnt signalling is transduced across the plasma membrane by the Frizzled (Fzd) receptor and a low density lipoprotein receptor-related protein (LRP). In the absence of signalling, free-cytosolic β -catenin is kept inactive by phosphorylation through a complex of glycogen synthase kinase-3 β (GSK-3 β) and the cytosolic protein Axin which sequesters the β -catenin in the cytoplasm through the ubiquitin pathway which marks it for proteosomal degradation (Fodde & Brabletz 2007). Activation of Fzd by Wnt ligands results in phosphorylation of GSK-3 β and sequestration of Axin to the plasma membrane. When GSK-3 β is unable to complex with Axin and it fails to phosphorylate β -catenin, which can then translocate to the nucleus to act as an important pro-EMT nuclear transcription factor (Fuxe & Karlsson 2012; Kim, K, Lu & Hay 2002; Medici, Hay & Olsen 2008; Sarkar, FH et al. 2010) (Figure 2.20). Just to add complexity, β -catenin has a molecular size of 90KDa which prevents its passive diffusion through the nuclear pores from the cytoplasm. Moreover, it does not contain nuclear localization signal (NLS) required for nuclear transport. There is quite a diversity among studies regarding proposed exact mechanisms of β -catenin transport into and inside the nucleus. However, evidence has emerged most strongly that β -catenin can directly interact with nuclear pore proteins to influence its own translocation to the nucleus (Fagotto, Glück & Gumbiner 1998). Further, LEF/TCF transcription factors can enrich β -catenin level in the nucleus (Huber et al. 1996), and a complex of B-cell lymphoma 9 (BCL9) and its nuclear interactor Pygopus can also strongly recruit β -catenin to that compartment (Kramps et al. 2002; Townsley, Cliffe & Bienz 2004). LEF-1 is a member of the lymphoid enhancer-binding factor/T-cell factor

(LEF/TCF) family of DNA-binding transcription factors, which interact with nuclear β -catenin and act as central transcriptional mediators of Wnt signalling (Clevers 2006).

Königshoff and colleagues demonstrated a role of the canonical Wnt pathway, outlined above, as a mediator for TGF- β signalling in activating alveolar type II cells to engage in the pathogenesis of pulmonary fibrosis (Königshoff et al. 2008; Königshoff & Eickelberg 2010). The reality of the mechanisms involved, however, are even more complex; e.g. a study by Medici et al, highlighted the importance of lymphoid-enhancer binding factor-1, yet another transcription factor in EMT stimulation. It does this through making a complex with β -catenin, plus Snail, under TGF- β stimulation (Medici, Hay & Olsen 2008). β -catenin may be thought of as having a quite contrasting dual role in the EMT activation process. When it is actively sequestered as a cell-cell adhesion factor it stabilises the system away from EMT activation. In this physiological situation it is bound into the cadherin complex with α -catenin. On the other hand, when the cadherin complex breaks down and β -catenin is released it has the potential to act in concert with other transcription factors in the nucleus, as above. Furthermore, and adding yet more complexity, in addition to Wnt glycoproteins, IGF-II, Ras and integrin-linked kinase (ILK), all can stimulate β -catenin accumulation in the cytoplasm, as well as also increasing Snail availability, possibly again through GSK-3 β inhibition or by inhibition of other kinases to facilitate EMT (Guarino, Tosoni & Nebuloni 2009; Kalluri, R. & Neilson, E. G. 2003; Nathke 1999; Postma, D. S. & Timens, W. 2006; Willis & Borok 2007; Zavadil & Bottinger 2005).

As alluded to above, several layers of crosstalk between the TGF- β and Wnt signalling pathways have been identified; for example, it is apparent that LEF-1 may be activated by binding to either Smad proteins or β -catenin (Labbé, Letamendia & Attisano 2000; Nishita et al. 2000). Furthermore, activation of TGF- β in pancreatic carcinoma cells induced the gene expression Wnt-5A, a canonical activating Wnt ligand that induces EMT and mediates the invasive capacity of pancreatic tumor cells (Ripka et al. 2007). All of this multi-layered, interactive complexity makes the idea of a “magic bullet” anti-EMT drug somewhat unrealistic, unless β -catenin itself could perhaps be directly targeted at the same time as TGF- β .

2.11.3.C Hedgehog Pathway

Hedgehog (Hh) signalling constitutes another potentially important pathways associated with induction of EMT; it was first discovered in *Drosophila* larvae (Nusslein-Volhard & Wieschaus 1980). In vertebrates, members of the Hh family include sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) (Katoh & Katoh 2005; Marigo et al. 1995).

Hh signalling is via the Patch receptor which normally keeps Smoothed protein (SMO) in an inhibited state (Johnson et al. 1996; van den Heuvel & Ingham 1996). Binding of Hh ligand to Patch receptors release SMO from this inhibition, which then initiates an intracellular cascade (Gallet & Therond 2005) that aids in the cytoplasm to nuclear translocation of the transcription factor Gli, this regulates not only the expression of its own related genes such as Gli and Patch (Stecca & Ruiz 2010), but also Wnt and Snail as well i.e. it initiates yet another positively reinforcing cycle towards EMT (Li, X et al. 2006; Taipale & Beachy 2001) (Figure 2.20).

The Shh pathway has been observed to be activated during both the physiological development of the lung and lung repair from injury (Watkins et al. 2003). Shh knockout embryos exhibit severe defects in the lung development with reduced airway branching and tissue growth (Bellusci et al. 1997; Litingtung et al. 1998; Pepicelli, Lewis & McMahon 1998). In adult tissues, growing evidence has highlighted the importance of the Hh pathway in cell migration. Wang and colleagues demonstrated in Gli knockout mice, reduced expression of Snail and MMP9 and increased the expression of E-cadherin (Wang, K et al. 2010), i.e. an anti-EMT effect. Similarly, inhibition of the Hh pathway with cyclopamine, a steroid that deactivate SMO, inhibited EMT (Taipale et al. 2000).

Yang L and colleagues suggested involvement of Hh signalling of human cancers (Yang, L et al. 2010), different types of lung cancer show activation of the Shh pathway (Bermudez et al. 2013; Chi et al. 2006; Park et al. 2011; Watkins et al. 2003; Yuan et al. 2006). It is involved in the mesenchymal transition of tumour cells associated with invasion (Varnat et al. 2009)

Overexpression of the Shh ligand has also been observed to be increased in the lungs of patients with usual interstitial pneumonia (UIP), (Coon et al. 2006), cryptogenic pneumonia (Stewart, GA et al. 2003) or IPF (Bolanos et al. 2012; Cigna et al. 2012).

Shh was expressed in bronchial and alveolar epithelial cells in fibrotic areas while Patch was observed to be up-regulated in fibroblasts, interstitial inflammatory cells and the hyperplastic epithelium of IPF patients (Bolanos et al. 2012; Cigna et al. 2012). These studies highlight the potential importance of the Hh pathway in lung fibrosis. Moreover, in fibroblasts, Shh activity was also reported to increase the synthesis of ECM proteins, collagen and fibronectin (Bermudez et al. 2013; Bolanos et al. 2012). Inhibition of the Hh pathway reduced expression of EMT-associated α -SMA, as well as collagen 1 and fibronectin end-products, and also downregulated the effects of TGF- β on these products (Cigna et al. 2012), again emphasising the complex cross-talk involved between pro-EMT pathways.

2.11.3.D uPAR pathway

The urokinase receptor (uPAR) has been reported to have an important role in EMT in the distal airways of COPD patients and its expression correlated with the degree of airflow obstruction and EMT activation of small airway epithelial cells (Wang, Q et al. 2013).

Urokinase was first isolated as a “clot-buster” from human urine and indeed is a modulator of the plasminogen pathway. Urokinase causes cleavage and activation of urokinase-type plasminogen activator (uPA) (Collen 1999). uPA- Upar axis is involved in the proteolytic activation of plasminogen into plasmin, which in turn activates matrix metalloproteases and degrades fibrin and other ECM components. The system, however, is much more ubiquitous than restricted to haematology: thus, binding of uPA to uPAR on epithelial cell membranes can also induce EMT by downstream activation of a number of cell-signalling enzymes, including PI3k, Akt, MAPK and myosin light chain kinase (Chandrasekar et al. 2003; Jo et al. 2009; Lester et al. 2007).

PI3k activation catalyses the formation of phosphatidylinositol 3, 4, 5-phosphate, which can influence cell morphology through cytoskeleton reorganization and migration (Chandrasekar et al. 2003). Another mechanism by which PI3k may also be involved in EMT is through the activation of Akt, which can promote cell invasion and regulate the activity of several transcription factors, including NF κ B (nuclear factor kappa B); NF κ B in turn, as transcription factor, can directly/indirectly induce the EMT gene programme, probably through intermediate transcription factors Snail1 or ZEB-1/2 (Larue & Bellacosa 2005).

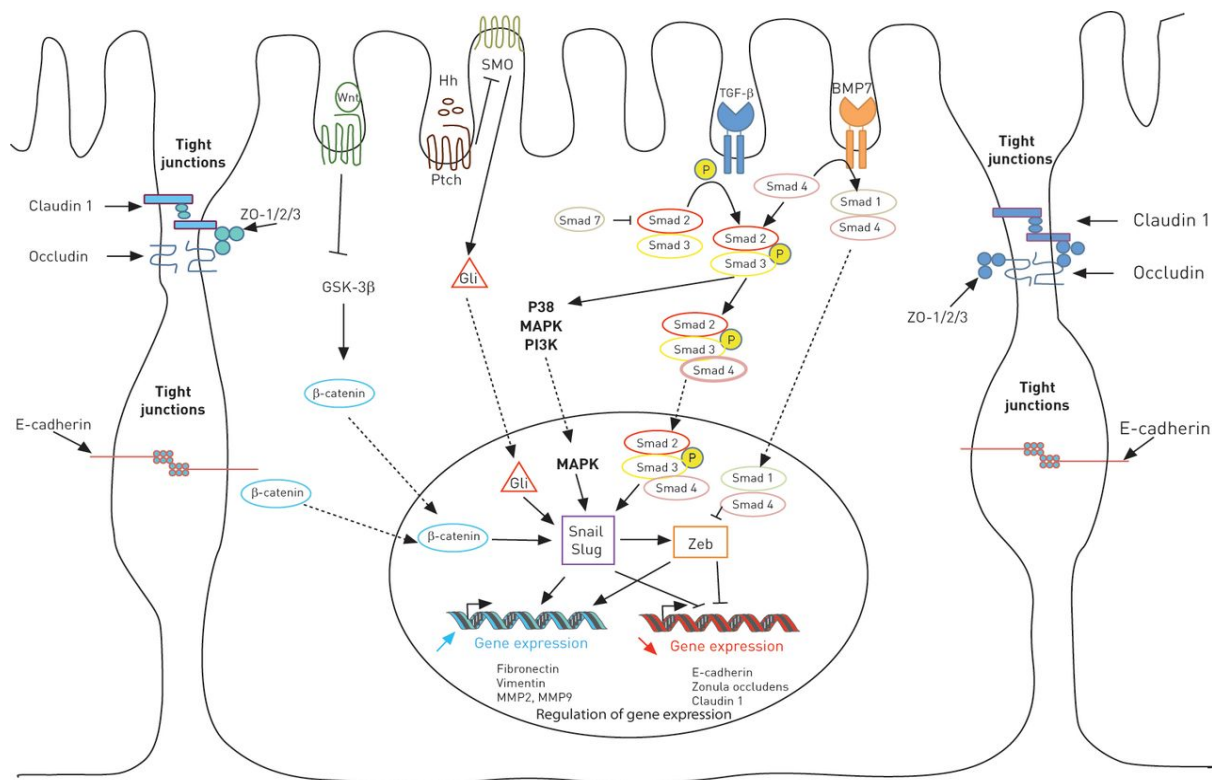


Figure 2-20: Cell signalling involved in epithelial–mesenchymal transition.

ZO: zonula occludin; Wnt: wingless tail; Hh: Hedgehog; SMO: Smoothened; GSK-3β: glycogen synthase kinase-3β; Ptch: Patched; MAPK: mitogen activated protein kinase; PI3K: phosphatidylinositol 3-kinase; Zeb: zinc finger E box binding homeobox; MMP: matrix metalloproteinase; TGF: transforming growth factor; BMP7: bone morphogenetic protein 7; P: phosphate.

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2.11.3.E Notch signalling pathway

The Notch signalling pathway maintains a balance between cell differentiation, proliferation and survival/apoptosis, and thus it is believed to play an important role in the development and progression of several malignancies; it also likely affects the development and function of many organs.

Notch signalling is activated by ligand-receptor binding which initiates an intercellular communication system. The trans-membrane receptors of Notch family, consists of four members: Notch-1-4. Notch receptors include an extracellular (ECD) and intracellular domain (ICD) (Fig 2.7). The Notch-receptor family ligands include Dll-1 (Delta-like 1), Dll-3 (Delta-like 3), Dll-4 (Delta-like 4), Jagged-1(JAG1) and Jagged-2(JAG2) (Miele 2006; Miele, Miao & Nickoloff 2006; Miele & Osborne 1999).

Notch ligand binding induces conformational change in the Notch receptor, leading to receptor activation and resultant receptor cleavage, involving a cascade of proteolytic steps by the metalloprotease tumour necrosis factor- α -converting enzyme (TACE) and γ -secretase complex. This cleavage of the Notch receptor results in release of its intracellular domain (ICN) and then its translocation of the ICN to the nucleus (Andersson, ER & Lendahl 2014; Miele 2006; Miele, Miao & Nickoloff 2006). In the nucleus, ICD binds with the CBF-1/suppressor of hairless/Lag1 (CSL) transcription factor and modulates downstream target gene expression (Figure 2.21). Important Notch target genes include NF- κ B, cyclin D1, c-myc, Akt, and VEGF and the transcription factor GATA3, all implicated in lung cancer biology (Fang et al. 2007; Miele 2006; Miele & Osborne 1999). Therefore, blocking these proteolytic enzymes could be potentially useful for the treatment of human malignancies.

The Notch signal pathway, activated by JAG-1, has been found to be a key regulator for the induction of EMT through Slug-induced repression of E-cadherin (Leong et al. 2007), the induction of hypoxia-inducible factor 1 α (HIF-1 α) (Sahlgren, C. et al. 2008), and the related process of endothelial-mesenchymal transition (Endo-MT) (Niessen et al. 2008; Sahlgren, C. et al. 2008; Timmerman et al. 2004; Zavadil et al. 2004). In the latter context, Endo-MT induces down- regulation of endothelial markers (VE-cadherin, Tie1, Tie2, endothelial NO synthase and platelet-endothelial cell adhesion molecule-1), up-regulation of mesenchymal markers (fibronectin, α -SMA, and platelet-derived growth factor receptors), and cell migration.

In addition to the direct effects mediated by its ICN, Notch system activation can also indirectly regulate EMT through influencing various parallel signalling pathways including NFkB and β -catenin, and through the action of various regulatory micro RNA (miRNAs) (Yang, Y et al. 2011). It has been observed that inhibition of Notch receptor activity in pancreatic cancer cell lines decreases the DNA binding potential of NFkB and lowers the expression of MMP9 (involved in ECM remodelling) and VEGF (involved in tumour invasion and metastasis), resulting in inhibition of pancreatic cancer cell invasion (Wang, Z et al. 2006). This system is complex and requires further in depth experiments to explore the actual mechanism involved.

Studies have shown that EMT activation is associated with profound suppression of micro-RNA (miR)-200 levels, while in mice forced expression of miR-200 locks tumor cells into an epithelial state and abrogates their metastatic capacity (Gibbons et al. 2009). Yang et al also reported that binding of the Notch ligand, JAG2 promotes EMT through inhibition of the miR-200 family via induction of GATA-binding protein 3 (a GATA family transcriptional factor) (Yang, Y et al. 2011).

A recent report by Hu and colleagues also observed cigarette smoke (CigS)-induced upregulation of Notch receptors (Notch 3) and ligands (DII2) in mice which seemed to play a critical role in pathogenesis of lung fibrosis (Hu, X-F, Zhang & Li 2014). Similarly, in NSCLC, Notch1 receptor activation was also observed to regulate the expression of Sox9 (a transcription factor) at its gene transcription promoter level, so by inducing repression of E-cadherin and leading to increased cell mobility and cell invasion (Capaccione et al. 2014).

In general, EMT processes involving the Notch pathway are regulated by its cross-talk with several core growth and transcription factors inherent to EMT, including Snail, Slug, TGF- β , FGF, and PDGF (Gonzalez & Medici 2014). Thus, inhibition of Notch signalling led to partial reversal of EMT through decreased expression of these transcription factors (Wang, Z et al. 2009).

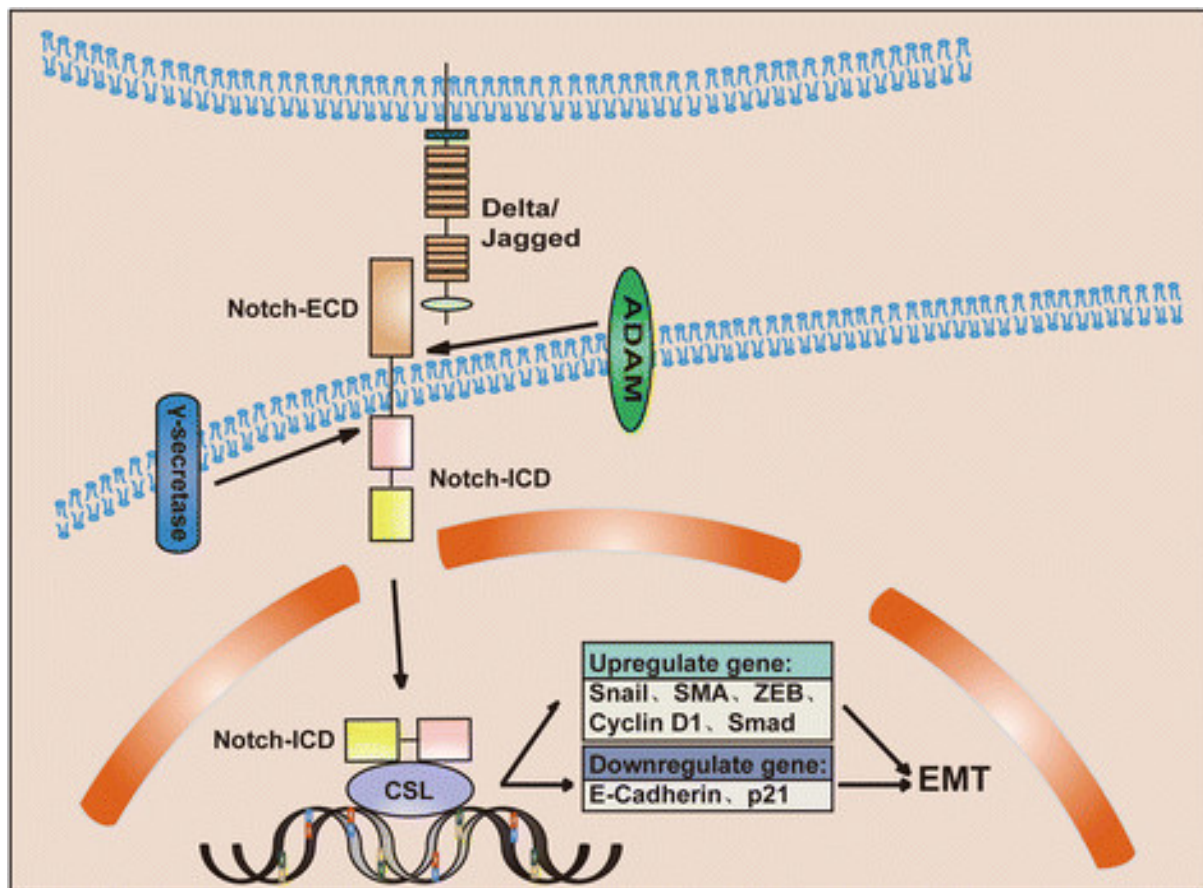


Figure 2-21: Schematic representation of Notch signalling related to EMT.

Upon ligand binding, Notch (receptor) undergoes two proteolytic cleavages by a combined ADAM and γ -secretase complex, leading to the release of Notch-ICD and its translocating to the nucleus. In the nucleus Notch-ICD interacts with CSL *and thereby influences a number of strategic* downstream target genes: upregulation of Snail, Slug, Zeb1, CyclinD1 and Smads; downregulation of E-cadherin and p21, so as to induce EMT. (Notch-ECD, the extracellular domain of Notch; Notch-ICD, the intracellular domain of Notch; ADAM, a disintegrin and metalloproteinase.

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2.11.4 Transcriptional regulation of EMT

EMT is complex pathophysiological process that involves multiple potential drivers, receptor systems and down-stream intracellular pathways, but also transcriptional factors. Most have been mentioned, but I will now deal with them specifically, with the exception of β -catenin as a separate entity, as I have discussed this extensively above as part of Pathways, but it will also appear in several other places in this section. The important transcription factors (TF) include the zinc-finger binding factors Snail1 (also called Snail) and Snail2 (also known as Slug) and several basic helix-loop-helix (bHLH) factors such as zinc finger E-box-binding homeobox 1 (ZEB1), ZEB2, and Twist (Peinado, Portillo & Cano 2004; Yang, J et al. 2004). Another highly efficient T cell factor (TCF) TF family member called lymphoid enhancer binding factor-1 (LEF-1) is also reported to directly induce EMT (Nawshad & Hay 2003). The modulation of binding of TFs or combinations of them to the promoter region of EMT-associated genes, as stimulators or repressors of transcription, is the ultimate end-product of the up-stream mechanisms discussed above. I will also try and draw out some clinically-relevant observations around expressions of these TFs in disease.

2.11.4.A Snail Family

The Snail family of transcriptional repressors plays a vital role in regulating EMT (Figure 2.22).

Of the multiple signalling pathways cooperating in the initiation and progression of EMT, a majority of them activate Snail1 expression. Notch, WNT family proteins, TGF β and growth factors that act through RTKs (receptor tyrosine kinase), can all activate Snail1 expression depending on the physiological context of tissue (Peinado, Olmeda & Cano 2007). Snail1 and Snail2 have mutual association with other transcription regulators to control gene expression. For example, Snail1 cooperates with the TF ETS1, which is activated by MAPK, to activate MMP expression (Jorda et al. 2005). It also cooperates with the Smad3 & 4 complex to cause the TGF β -mediated repression of E-cadherin and zona-occludin expression (Vincent, T. et al. 2009) to cause release of β -catenin from these complexes (Nawshad & Hay 2003; Peinado, Portillo & Cano 2004). Reciprocally, the Wnt- β -catenin and PI3k-AkT mechanism also increase Snail1

activity by preventing its phosphorylation by GSK-3 β , which enhances EMT (Yook et al. 2006).

Various studies have shown that Snail1 induces the expression of mesenchymal proteins like N-cadherin, fibronectin, vitronectin and MMPs (Barrallo-Gimeno & Nieto 2005; Olmeda et al. 2007), at least partially through enhanced β -catenin pro-transcription activity, that further encourage Snail1 accumulation, and so its availability as a pro-EMT transcription factor (Medici, Hay & Olsen 2008).

These mechanisms have major clinical relevance. Thus, overexpression of either Snail1 or Snail2 in tumour cells induces EMT and correlates with increased tumor metastasis in vivo, consistent with the concept of developmental reprogramming reactivated in metastatic carcinomas. (Batlle et al. 2000; Cano et al. 2000). Thus, Snail1 accumulation in the nucleus of breast cancer cells, associated with decreased cellular E-cadherin expression, has been reported to induce metastatic phenotypes (Yook et al. 2006). Similarly, circulating tumour cells isolated from patients with metastatic hepatocellular carcinoma (HCC) were found to have 20 times more Snail1 expression than cells isolated from patients with non-metastatic HCC (Min et al. 2009).

Snail2 (Slug) has a prominent role embryologically in the induction of EMT in gastrulation and for development and migration of the neural crest, but has also been reported as involved in cancer metastasis (Thiery, Jean Paul et al. 2009; Yang, J & Weinberg 2008).

2.11.4.B E47 and Twist

Basic helix–loop–helix (bHLH) TFs function as master regulators of lineage specification and differentiation. Among these, E47, Twist1 and Twist2, proteins have key roles in EMT progression (De Craene & Berx 2013; Peinado, Olmeda & Cano 2007). Like Snail, Twist expression not only downregulates expression of epithelial genes but also activates and upregulate mesenchymal gene expression (Xu, J, Lamouille & Derynck 2009) (Figure 2.22). In cancer cells, Twist1 was observed to represses E-cadherin and induce N-cadherin expression independently of Snail, probably through the association with other TF proteins (Xu, J, Lamouille & Derynck 2009; Yang, F et al. 2012; Yang, MH et al. 2010; Yang, MH et al. 2008).

Multiple signalling pathways regulate Twist expression during development and disease, especially in malignant settings (Peinado, Olmeda & Cano 2007; Xu, J, Lamouille & Derynck 2009). Yang et al reported that an important transcription factor, hypoxia-inducible factor 1 α (HIF1 α) induced Twist expression under hypoxic conditions, so promoting EMT and ultimately dissemination of tumour cell (Yang, MH et al. 2008). Moreover, mechanical stress induced Twist expression, dependent on β -catenin, in *Drosophila melanogaster* epithelia (Farge 2003).

Twist has been associated with fibrotic pathology in many organs including kidney and liver (Iizuka et al. 2012; Sun et al. 2009). Twist-associated pulmonary fibrosis was first demonstrated in a murine model of virus-induced lung fibrosis and in alveolar epithelial cells from lung tissue of IPF patients (Pozharskaya et al. 2009).

Twist over-expression has been detected in many tumours, including breast, prostate, and hepatocellular carcinoma, and inhibition of Twist expression significantly improve the survival of patients with carcinoma (Lee, TK et al. 2006; Wushou et al. 2014). However, the prognostic significance of Twist expression in lung cancer remains controversial. Miura et al. suggested that Twist was not associated with EMT and might not predict the survival of NSCLC (Miura et al. 2009). However, later, Hung et al suggested an inverse correlation between Twist expression and patient prognosis (Hung et al. 2009) which was further supported by a recent study by Zeng et al who described Twist as a poor prognostic indicator but still potential target for treating lung cancer (Zeng et al. 2015).

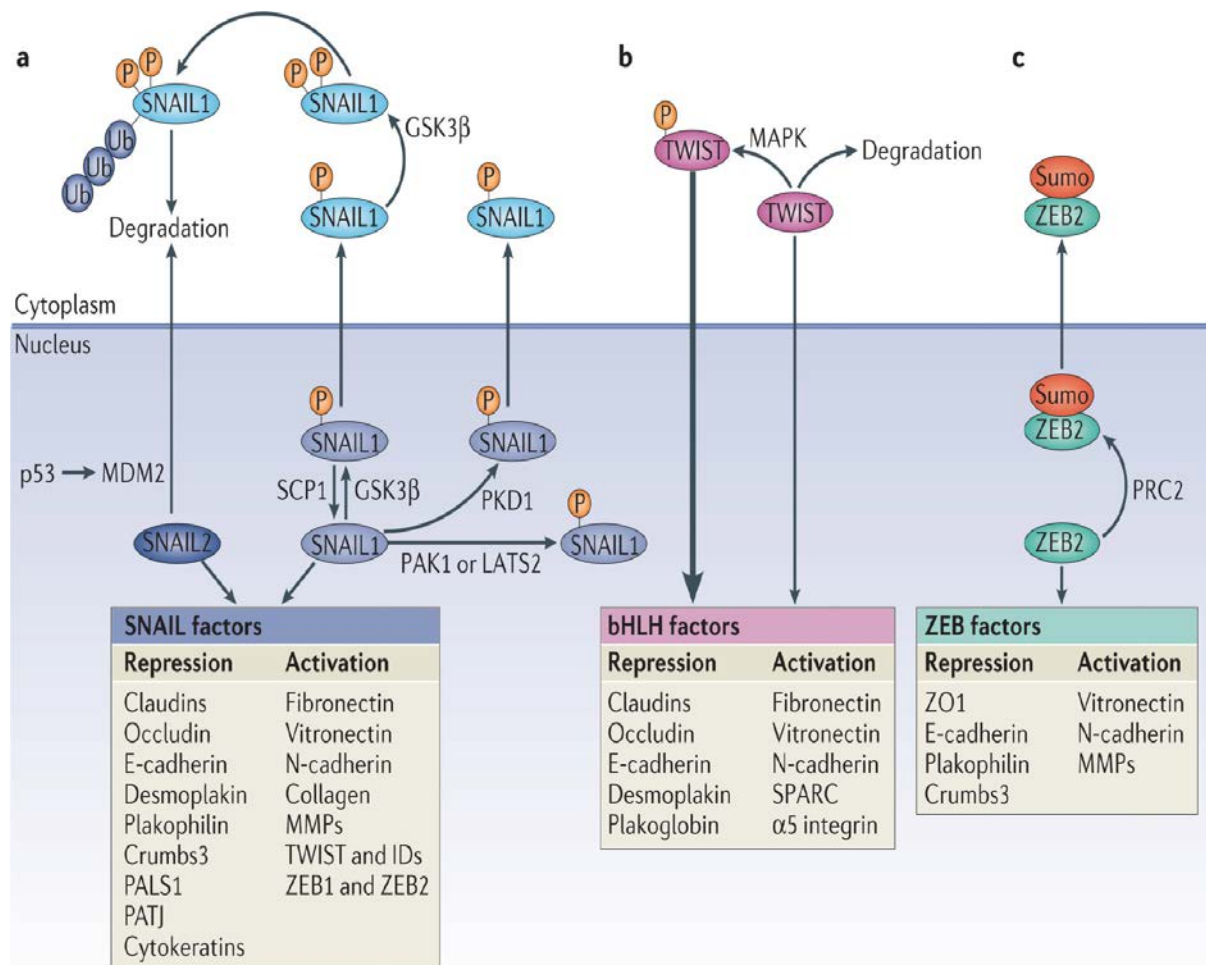


Figure 2-22: Regulation of transcriptional factors in EMT.

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2.11.4.C ZEB TF Family

The ZEB family (ZEB1 and ZEB2) are transcriptional repressors with an essential role in neural crest development and have been studied as key regulators of cancer progression (Van de Putte et al. 2003). ZEB1 and ZEB2 interact with the E-box regions of DNA to cause the CDH1 gene to repress its promoter activity for E-cadherin transcription (Eger, Andreas et al. 2005; Remacle et al. 1999). Expression of ZEB in epithelial cells was shown to be sufficient to induce the dissociation of adherens junctions, presumably through suppressing the expression of relevant genes, and so again releasing the central player in EMT induction, β -catenin (Eger, Andreas et al. 2005; Vandewalle et al. 2005). Their expression also increased the expression of genes encoding matrix metalloproteinases (MMPs), implicating ZEB1 and ZEB2 in matrix remodelling and movement of transitioning cells through tissue (Miyoshi et al. 2004) (Figure 2.22).

ZEB expression often follows activation of Snail expression, consistent with Snail1 directly targeting the ZEB1 gene. Additionally, Twist1 cooperates with Snail1 in this (Dave et al. 2011). ZEB expression is also regulated by TGF β and Wnt proteins, and growth factors that activate Ras-MAPK signalling (Xu, J, Lamouille & Derynck 2009). The induction of ZEB expression by TGF β signalling involves ETS1, which is activated by MAPK signalling (Shirakihara, Saitoh & Miyazono 2007).

ZEB1 has been implicated in many malignancies, especially NSCLC where it is reported to be involved in tumor progression by repressing the tumour suppressor gene (SEMA3F) and represents an important potential therapeutic target (Clarhaut et al. 2009). A recent study by Nishijima and colleagues, investigated reversal of TGF β -1 induced EMT by Nintedanib (Anti-EGFR) and also reversing resistance to Gefitinib by downregulating ZEB1 as well as miR-200b and miR-141 upregulation. They suggested that the miR-200/ZEB axis of EMT might be a predictive biomarker for sensitivity to Nintedanib in NSCLC cells (Nishijima et al. 2016).

MicroRNAs (miRs) are single-stranded, non-coding small RNA molecules, 18–24-nucleotide in length that bind to the 3' un-translated regions (UTRs) of their target messenger RNAs (mRNAs), thereby, repressing their translation as well as inducing their degradation (Stefani & Slack 2008). The first such identified miRNAs in 1984 were, the products of the *C. elegans* genes (*lin-4* and *let-7*), were later reported to

have an important role in controlling developmental timing (Ambros & Horvitz 1984; Lee, RC, Feinbaum & Ambros 1993; Reinhart et al. 2000).

miRs are involved in regulation of numerous molecular and cellular processes. Aberrant expression of miRs have been associated with initiation and progression of pathological processes, including diabetes, cancer, and cardiovascular disease (Croce 2009; Latronico & Condorelli 2009; Pandey et al. 2009; Thum et al. 2008). In addition, there is mounting evidence that shows miRNAs are mutated or poorly expressed in human cancer, suggesting that miRNAs may normally act as tumor suppressors or oncogenes (Calin et al. 2002; McManus 2003; Takamizawa et al. 2004). Growing evidence also suggests that miRNAs participate in the fibrotic process in a number of organs including the heart, kidney and liver (Jiang, X et al. 2010). For example, three important TGF- β -regulated miRNA families, miR-200, miR-21, and miR-29 have been shown to modulate fibrosis in kidney. These three miRs work in opposite direction to each other, with miR-21 amplifying TGF- β signalling and promoting fibrosis, while miR-200 and miR-29 reduce fibrosis by inhibiting EMT and preventing ECM deposition, respectively. Augmenting miR-29 expression or inhibition of miR-21 expression, prevented renal fibrosis in mice (Patel & Nouredine 2012).

The miR-200 family consists of five members: miR-200a, miR-200b, miR-200c, miR-429, and miR-141. miR-200 is enriched in kidney and lung, where its functional importance is to maintain epithelial differentiation (Patel & Nouredine 2012). However little is known regarding the role of miRNAs in lung fibrosis, although it is in a position to be quite strategic. Yang et al have observed that in a mouse model, expression of miR-200 was decreased in lung fibrosis, and introduction of miR-200c in mice, diminished pulmonary fibrosis (Yang, S et al. 2012).

The mechanism of the anti-fibrotic effect of miR-200 is likely to involve prevention of EMT (Korpai et al. 2008). A study by Gregory et al of in-vitro assays reported that miR-200s prevent TGF- β -mediated EMT through repression of Zeb1 and Zeb2 (Gregory et al. 2008). This evidence provides basis for making miR-200/Zeb axis to be an important predictive biomarker for Nintedanib sensitivity in NSCLC cells as well (Nishijima et al. 2016). In depth and detailed explanation of miRNA is beyond the scope of my thesis.

In contrast, with a different miR, Pottier et al observed that miR-155 expression was increased in human lung fibroblasts following treatment with TNF α and IL-1 β and reduced by TGF- β (Pottier et al. 2009), and in bleomycin-induced lung fibrosis model in mice, they confirmed that up-regulation of miR-155 was correlated with the degree of lung fibrosis.

2.11.5 Cell and Tissue Markers of EMT

Fundamental outcomes during EMT are, strategic protein acquisition and suppression with, the conversion of epiblasts to primary mesenchyme (Carver et al. 2001; Ip & Gridley 2002) or epithelium to fibroblasts (Zavadil, J. et al. 2001), or in the transition of tumorous in-situ epithelium cells into metastatic cells (Ramaswamy et al. 2003), in the context of the three distinctive types of EMT. Initially, studies generally intended to focused on only one or two event markers (for example, changes in E-cadherin or Snail expression) but with passage of time, better understanding of the complexity of the EMT process and with fuller description of the spectrum of protein expression from epithelium to fibroblast (Kim, K, Lu & Hay 2002; Nieto 2002) (Figure 2.23), made it now possible to formulate extensive criteria for recognition of EMT activity in tissue samples (Figure 2.24). This has allowed our group, for example, to be able to designate EMT as present and active in airway biopsies and resected tissue with some confidence.

Markers of EMT

| Acquired markers | | Attenuated markers | |
|------------------------------|----------|-------------------------|----------|
| Name | EMT type | Name | EMT type |
| Cell-surface proteins | | | |
| N-cadherin | 1, 2 | E-cadherin | 1, 2, 3 |
| OB-cadherin | 3 | ZO-1 | 1, 2, 3 |
| $\alpha 5\beta 1$ integrin | 1, 3 | | |
| $\alpha V\beta 6$ integrin | 1, 3 | | |
| Syndecan-1 | 1, 3 | | |
| Cytoskeletal markers | | | |
| FSP1 | 1, 2, 3 | Cytokeratin | 1, 2, 3 |
| α -SMA | 2, 3 | | |
| Vimentin | 1, 2 | | |
| β -Catenin | 1, 2, 3 | | |
| ECM proteins | | | |
| $\alpha 1(I)$ collagen | 1, 3 | $\alpha 1(IV)$ collagen | 1, 2, 3 |
| $\alpha 1(III)$ collagen | 1, 3 | Laminin 1 | 1, 2, 3 |
| Fibronectin | 1, 2 | | |
| Laminin 5 | 1, 2 | | |
| Transcription factors | | | |
| Snail1 (Snail) | 1, 2, 3 | | |
| Snail2 (Slug) | 1, 2, 3 | | |
| ZEB1 | 1, 2, 3 | | |
| CBF-A/KAP-1 complex | 2, 3 | | |
| Twist | 1, 2, 3 | | |
| LEF-1 | 1, 2, 3 | | |
| Ets-1 | 1, 2, 3 | | |
| FOXC2 | 1, 2 | | |
| Goosecoid | 1, 2 | | |
| MicroRNAs | | | |
| miR10b | 2 | Mir-200 family | 2 |
| miR-21 | 2, 3 | | |

ZEB1, zinc finger E-box binding homeobox 1.

Figure 2-23: Markers of EMT.

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In vivo criteria for EMT

Major criteria

- Use of an epithelial cell reporter construct that appears locally in newly formed fibroblasts
- New expression of FSP1 and possibly DDR2 associated with disruption of basement membrane
- Increased expression of HSP47, collagen I ($\alpha 1$), collagen 2 ($\alpha 2$), N-cadherin, or vimentin
- Nuclear relocalization of CBF-A or β -catenin/LEF or new expression by in situ hybridization of one of the following transcription factors: Snail, Slug, or Twist
- Loss or partial loss of epithelial markers such as cytokeratin, E-cadherin, or ZO-1
- Spindle-shape morphology with redistribution of stress fibers and loss of polarity

Minor criteria

- Localized adjacency of transitioning cell near its epithelial compartment
- Exclusion of possible bone marrow-derived progenitor cells

In vitro criteria for EMT

Major criteria

- New expression of FSP1 and possibly DDR2
- Increased expression of HSP47, collagen I ($\alpha 1$), collagen 2 ($\alpha 2$), or vimentin
- Cadherin switch
- Nuclear relocalization of CBF-A or β -catenin/LEF or new expression of one of the following transcription factors: Snail, Slug, or Twist
- Absence of epithelial markers; loss of cytokeratin or ZO-1
- Spindle-shape morphology with redistribution of stress fibers and loss of polarity
- Resistance to apoptotic stimuli
- Increased migratory capacity
- Phenotype stable upon removal of inducing stimulus

Minor criteria

- Abundant intermediate filaments and microfilaments
- Loss of chromatin condensation associated with gain of multiple nucleoli
- Gain of rough ER, abundant lysosomal granules, and loss of intercellular junctions on electron microscopy

Figure 2-24: In vivo and in vitro criteria from EMT.

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2.11.5.A S100A4 (FSP-1)

S100A4 (Fibroblast specific protein-1, FSP-1) is 10-12 kDa protein containing 101 amino acids (Vallely et al. 2002), and a member of the S100 calcium-binding protein family (Santamaria-Kisiel, Rintala-Dempsey & Shaw 2006; Tarabykina et al. 2007). In wide ranges of cells including stromal and tumour cells, S100 proteins are localized in the cytoplasm and/or nucleus. S100A4 (FSP-1) is involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100A4 (FSP-1) is also associated with multiple functions as motility, tubulin polymerization and so tumour invasion.

S100A4 (FSP-1) is not typically present in normal epithelial cells, but is now a classic early and important cytoskeletal marker of EMT, associated with activities such as cell motility, angiogenesis, smooth muscle cell (SMC) migration and proliferation, (Ambartsumian et al. 2001; Bowers et al. 2012; Iwano, M. et al. 2002; Lawrie et al. 2005; Zeisberg et al. 2003; Zeisberg, M et al. 2007). Thus, S100A4 (FSP-1) expression by epithelial cells is usually taken as a marker that they are undergoing transition to mesenchymal-type cells during tissue fibrosis in EMT Type 2 (Zeisberg & Neilson 2009), and epithelial cells positive for S100A4 (FSP-1) serve as a precursor to a proportion of fibroblasts expressing S100A4 found in fibrogenic foci of affected tissue (Iwano, M. et al. 2002). Further mesenchymal cells, produced in the EMT process have strong migratory abilities through expressing S100A4 (FSP-1) (Lee, J. M. et al. 2006; Thiery, J. P. & Sleeman 2006).

In the setting of malignancy, S100A4 (FSP-1) supports tumorigenesis by stimulating angiogenesis in synergism with VEGF to induce endothelial cell migration via stimulating RAGE (an advanced glycosylation end product-specific) receptor, making S100A4 (FSP-1) an attractive potential therapeutic target in human cancer (Hernandez et al. 2013). Indeed, chromosomal re-arrangements and altered expression of the S100A4 (FSP-1) gene have been implicated in tumour metastasis, and a recent Study by Dahlmann et al reported that tumour metastasis involved RAGE receptors and S100A4 mediated cell motility through MAPK/ERK activation (Dahlmann et al. 2014).

Similarly, in a cancer model, metastatic tumour cells expressing S100A4 (FSP-1), indicated a molecular re-programing associated with invasiveness in EMT Type 3 (Kalluri, Raghu & Zeisberg 2006).

There is also emerging evidence that S100A4 (FSP-1), along with rendering migratory capacity to mesenchymal cells, can also activate and release MMPs (matrix metalloproteinases) that also play a leading role in tissue remodelling in established EMT (Korol et al. 2014). The mechanisms of MMP regulation by S100A4 (FSP-1) are mostly unknown, but recent studies suggest that many extracellular activity is involved (Schmidt-Hansen et al. 2004; Yammani et al. 2006) whose detail is beyond the scope of this thesis. However, in cancer like osteosarcoma, down-regulation of S100A4 (FSP-1) expression in malignant cells led to reduced expression of MT1-MMP and MMP-2, with a subsequent reduction in MMP activity and a reduced ability to migrate (Bjornland et al. 1999). In human prostate cancer, cancer cells were also observed to be stimulated by S100A4 (FSP-1), at least partly through transcriptional activation of MMP-9 (Saleem, M. et al. 2006). Moreover, in vivo and in vitro studies shows that gene manipulations cause induction of S100A4 (FSP-1) expression which render invasive capabilities to colorectal cancer cell line (Stein et al. 2006).

This functional capacity of transitioned mesenchymal cells, associated with S100A4 (FSP-1) expression is important for progression of various diseases (Lee, J. M. et al. 2006; Thiery, J. P. & Sleeman 2006). Thus, EMT has also been implicated in organ fibrosis (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2011; Xu, X et al. 2014). Similarly, in IPF patients, strong staining for S100A4 (FSP-1) have been observed in disease-associated fibroblasts (Schneider, Hansen & Sheikh 2008). S100A4 (FSP-1) localization to bronchial epithelial cells in human transplanted lungs were thought indicative of EMT in the airways leading to remodelling and fibrosis as part of chronic rejection (Hodge et al. 2009; Ward, C. et al. 2005). Lawrie et al. showed that in pulmonary vascular disease, smooth muscle cells of pulmonary vessels, proliferation and migration was dependent upon the activation of RAGE receptors by S100A4 (FSP-1) (Lawrie et al. 2005).

A significant increase in S100A4 (FSP-1) expression has also been found in NSCLC (Chen, XL et al. 2008) related to the S100A4/MMP9 signalling axis promoting invasive capacity. In patients with lung adeno carcinoma, S100A4 expression is associated with reduced overall survival. This study also demonstrated that Niclosamide (an anti-

helminthic drug) induced suppression of S100A4 (FSP-1), and so inhibited the invasive and metastatic capacity of NSCLC (Stewart, RL et al. 2016).

Tissue fibrosis and cancer share common molecular and biological mechanism, signalling pathways, especially associated with EMT. All these evidences make S100A4 (FSP-1) a potential therapeutic target for prevention/treatment of both fibrosis and cancer by ameliorating EMT mechanism (Hodge et al. 2009; Okada et al. 1997; Saleem, M. et al. 2006; Schneider, Hansen & Sheikh 2008; Ward, C. et al. 2005; Zeisberg & Neilson 2009).

2.11.5.B Vimentin

Vimentin is a 57 kDa type III intermediate filament protein normally expressed in cells of mesenchymal origin, also in epithelial cells as marker of them undergoing EMT (Kalluri, R. & Neilson, E. G. 2003). It is also involved in tumour invasion (Fuchs & Weber 1994). Vimentin abundance in epithelial cells undergoing EMT suggested a particular role in epithelial cell migration (Lee, J. M. et al. 2006).

Vimentin has been found to be expressed in a wide range of cell types including sertoli cells, macrophages, neutrophils, endothelial cells, fibroblasts, leukocytes, and renal tubular and mesangial cells but importantly not in normal differentiated epithelial cells (Carter et al. 2005; Evans 1998; Gilles et al. 1999; Kokkinos et al. 2007). Vimentin found in the mesenchymal cells is mostly cytoplasmic, where its filaments help to maintain cellular architecture and tissue integrity (Franke et al. 1982). Vimentin is upregulated by the transcription factor TCF/LEF, known to interact with a large number of proteins which participate in formation of complexes with cell signalling molecules (e.g. Ras/MAPK/Akt 1) and other adaptor proteins (e.g. Scrib/Slug/Axl) (explained below) (Perlson et al. 2006; Phua, Humbert & Hunziker 2009; Vuoriluoto et al. 2011).

In the EMT setting, vimentin is often associated with reduced E-cadherin expression at the cell membrane (Kokkinos et al. 2007) and as mentioned, reported to be functionally involved in migratory potential of transitioning epithelial cells (Bartel-Friedrich et al. 2007; Gilles et al. 1999). Vimentin is also reportedly involved in several other mechanisms associated with EMT (Vuoriluoto et al. 2011); including, (a) *Gene expression*: vimentin (indirectly) regulates gene expression of Axl 3 (tyrosine

kinase) proteins required for execution of EMT; (b) *Protein-protein interactions*: vimentin regulates anterior-posterior cell polarity and directional motility by directly binding and stabilizing Scribble (polarity-complex protein) (Mendez, Kojima & Goldman 2010; Phua, Humbert & Hunziker 2009); (c) *Phosphorylation*: vimentin can be phosphorylated by Akt1, (Ras/PI3k), which protects vimentin from caspase-induced proteolysis and promotes cell motility and EMT-related metastasis (Hoshino et al. 1999). Mesenchymal cells, e.g. fibroblasts, which lack vimentin migrate poorly (Eckes et al. 1998) and vimentin-deficient mice exhibit delayed wound healing (Eckes et al. 2000). Increased serum levels of vimentin were observed in IPF patients (Yang, Y et al. 2002). In an in-vitro model of IPF, vimentin was required for remodelling in alveolar epithelial cells (Rogel et al. 2011), suggesting an important role in pathogenesis of fibrotic lung diseases. In the context of post-lung transplant chronic rejection, vimentin has been reported to be increased in airway epithelium with coincident decreased E-cadherin (an epithelial marker) during EMT-induced airway remodelling (Borthwick et al. 2009).

Hackett et al found increased levels of vimentin in TGF- β -induced EMT in primary airway epithelial cell cultures from patients with asthma (Hackett, TL et al. 2009). Similarly, Sophie and colleagues reported that bronchial epithelium from COPD patients displayed increased vimentin expression and decreased epithelial differentiation, suggesting that the epithelium from COPD patients retain structural programming related to EMT even in-vitro (Gohy et al. 2013). Milara et al also observed in airway biopsies that, in comparison to non-smokers, currently smoking COPD patients had up-regulation of various mesenchymal protein including vimentin (Milara, Peiro, et al. 2013).

Vimentin expression in carcinoma cells correlated with increased invasiveness and metastasis. This has made vimentin a common marker for identifying EMT Type 3 in cancers (Zeisberg & Neilson 2009). In lung cancers, vimentin has been detected in moderately and well-differentiated adenocarcinomas and in giant cell carcinomas (Upton et al. 1986). Vimentin overexpression was found to be an independent predictor of poor survival in patients with resected non-small-cell lung cancer (Al-Saad et al. 2008). In another study by Rho et al, glycosylated vimentin was observed to be downregulated in human lung adenocarcinomas; glycosylated vimentin was thus suggested to represent a new functional biomarker for the diagnosis, and post-

treatment follow-up of patients with lung cancers (Rho, Roehrl & Wang 2009). Vimentin from normal tissue has sialic acid and terminal GlcNAc residues (monosaccharides), but vimentin in cancer tissue did not show these modifications which is the main reason of decreased expression of glycosylated vimentin in cancer. Furthermore, vimentin's overexpression in metastatic cancer suggests it also has a role as a metastasis promoter (Hu, L et al. 2004; Jin et al. 2010; Li, M et al. 2010; Wei, J et al. 2008).

2.11.5.C N-cadherin

N-cadherin is a 130 kDa molecule, first identified in chick neural retina in 1982 (Brundwald, Pratt & Lilien 1982), and later described as an adhesion molecule, localised to adherens junctions. (Volk & Geiger 1984). N-cadherin is also known as neural-cadherin, non-epithelial cadherin or cadherin-2. Along with ER-cadherin, N-cadherin belong to a family of calcium-dependant adhesion molecules, functioning to mediate the cell-to-cell binding that is required for the maintenance of morphogenesis and tissue cytoskeletal organization.

E and N-cadherin usually work in reciprocation to each other under a process called "cadherin switching". The term also includes situations in which cells turn on (or increase) expression of N-cadherin without significantly changing E-cadherin expression (Wheelock et al. 2008). This cadherin switching is very important for cell migratory capacity and invasiveness associated with mesenchyme-like cells produced as a result of EMT in fibrotic and malignant diseases.

N-cadherin activation/up-regulation involves multiple growth factor receptors (e.g. FGFR), enzymic proteins (e.g. MAPK/ERK and Rho-GTPases, RhoA), and other regulatory molecules such as the catenins, and in particular the p120 catenin (p120ctn), β -catenin and α -catenin (Doherty & Walsh 1996; Lilien et al. 2002; Suyama et al. 2002). In mesenchymal cells like fibroblasts, cytoplasmic p120ctn inhibits RhoA, resulting in an increase in cell motility (Magie, Pinto-Santini & Parkhurst 2002).

N-cadherin has been reported to be increased in lung tissue remodelling in IPF patients, with potential as a novel prognostic marker (Lomas et al. 2012). Similarly, in a COPD setting, N-cadherin was also reported to be overexpressed in smoking individuals in comparison to non-smokers, giving an additional clue that EMT is active in human bronchial epithelial cells of COPD patients, (Nishioka, M et al. 2015).

N-cadherin has been studied in a variety of epithelial malignancies including in pancreas, liver, colon, prostate, kidney and breast carcinomas (Behnes et al. 2012; Nakajima, S et al. 2004; Rezaei et al. 2012; Shimazui et al. 2006; Tran et al. 1999; Zhuo et al. 2013). In NSCLC, Nakashima T and other's indicated that the frequency of hyper vascular tumours was significantly higher for N-cadherin positive carcinomas than for N-cadherin-negative carcinomas and the 5-year survival rate of patients with N-cadherin- positive tumours was significantly lower than that of patients with N-cadherin-negative tumours (Nakashima et al. 2003).

Gefitinib, a tyrosine kinase inhibitors of the EGFR, has revolutionized molecularly targeted therapy in some but not all lung cancer patients. At the same time, some have acquired resistance after long-term treatment. Mai Yamauchi et al suggested that, N-cadherin may play a vital role in survival of Gefitinib-resistant lung cancer cells via the PI3k/Akt survival pathway (Yamauchi et al. 2011).

Hui et al observed that overexpression of N-cadherin (and Twist) could be useful as a biomarker for predicting the prognosis of NSCLC. They investigated a mutual association of Twist1 and N-cadherin whereby together they inhibited apoptosis and promoted the invasion of lung cancer cells, but depletion of Twist1 in lung cancer cells also led to inhibition of N-cadherin expression (Hui et al. 2013). Zhang et al highlighted N-cadherin upregulation in Erlotinib-resistant cell line and suggested this as responsible for increased tumour cell migration, invasion and tumorigenic potential and all related to sustained EMT expression. Therefore, N-cadherin may serve as a new target for the treatment of multiple cancers where acquired resistance to EGFR-TKIs (Epidermal growth factor Receptor-Tyrosine Kinase Inhibitors), is frequent (Zhang, X et al. 2013).

Thus in general, expression of N-cadherin clearly plays a role in the progression of transition from epithelium to mesenchyme in EMT in both malignant and non-malignant pathology. It is likely that N-cadherin not only mediates a less stable cell-

cell adhesion but allows cell invasion as well. Cancer cells derived from epithelium inappropriately express N-cadherin, which promotes motility and invasion. The disturbance of the adherens complex releases β -catenin, which was discussed earlier would escalate the drive towards EMT.

2.11.5.D EGFR: Epithelial activation marker

The epidermal growth factor receptor (EGFR), is a 170-kDa glycoprotein, a founding member of the ErbB receptor tyrosine kinase (RTK) family. The multifunctional role of murine epidermal growth factor (EGF) and its human equivalent, β -urogastrone have been studied extensively since first discovered by Stanley Cohen, about half a century ago (Cohen 1960). The RTK family consists of four transmembrane receptors including EGFR (also known as ErbB1/ HER-1), ErbB2/Neu/HER-2, ErbB3/HER-3, and ErbB4/HER-4 (Schlessinger 2002). EGFR ligands include epidermal growth factor (EGF), transforming growth factor- α (TGF- α), epiregulin (EREG) amphiregulin (AR), and heparin-binding EGF (HB-EGF).

The EGFR consists of an extracellular receptor domain, a transmembrane region with a single hydrophobic anchor sequence and an intracellular portion with tyrosine kinase function. Ligand binding to EGF receptors induces a complex conformational change with resultant activation of the intrinsic kinase domain and phosphorylation of specific tyrosine residues (PLC- γ , MAPK & GAP) within the cytoplasmic tail that leads to activation of downstream signalling cascades (including Ras/MAPK/STATs/PI3k-Akt), transducing extracellular stimuli to nuclear transcriptional factors (Schlessinger 2002; Voldborg et al. 1997; Yarden & Sliwkowski 2001) (Figure 2.25).

In the human lung, EGFR is normally located on airway epithelium cells, smooth muscle cells, and bronchial glands; where, they can be activated by ligands secreted by the epithelium and underlying mesenchyme, including smooth muscle cells (Aida et al. 1994; Amishima et al. 1998; Koff et al. 2008). Activation of the EGFR pathway is usually associated with local autocrine changes, for example epithelial wound repair and vascular smooth muscle cell proliferation, as well as paracrine signalling from the epithelium to the mesenchyme (Burgel & Nadel 2008; Ingram & Bonner 2006; Zhou, Ibe & Raj 2007). Although EGFR plays an important role in tissue morphogenesis and repair, dysregulation of this pathway is also implicated in various pathologic conditions.

Thus over-activation of the EGFR pathway has been observed in lung remodelling associated several chronic lung diseases, including pulmonary fibrosis, BPD (bronchopulmonary dysplasia), cystic fibrosis, asthma and COPD (Amishima et al. 1998; de Boer et al. 2006; Normanno et al. 2003; O'Donnell, RA et al. 2004; Stahlman, Orth & Gray 1989; Strandjord et al. 1995). In addition, duplications of the EGFR gene and active mutations in its tyrosine kinase domain have been found in several tumours, including NSCLC (Carlson, JJ et al. 2009; Charpidou et al. 2008) (Figure 2.26).

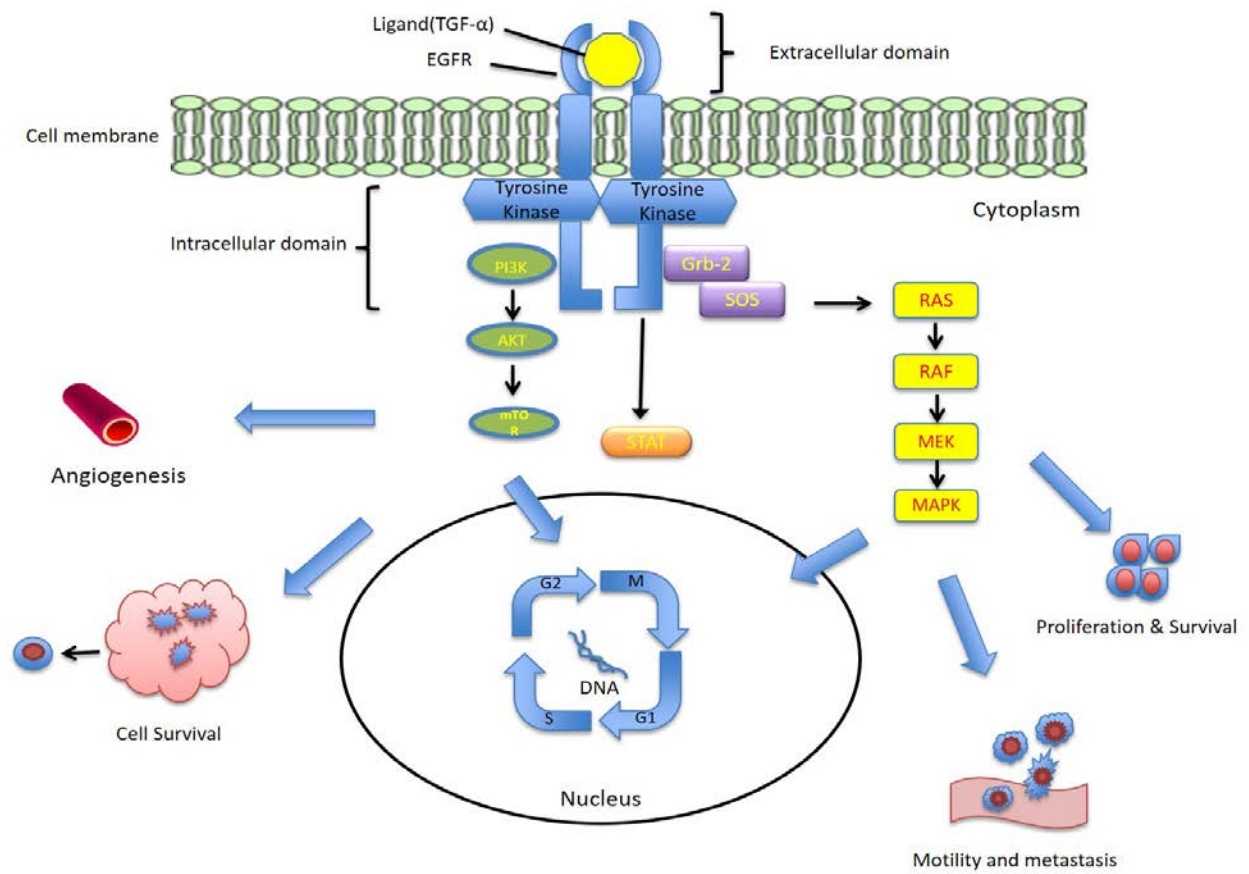


Figure 2-25: EGFR Pathway.

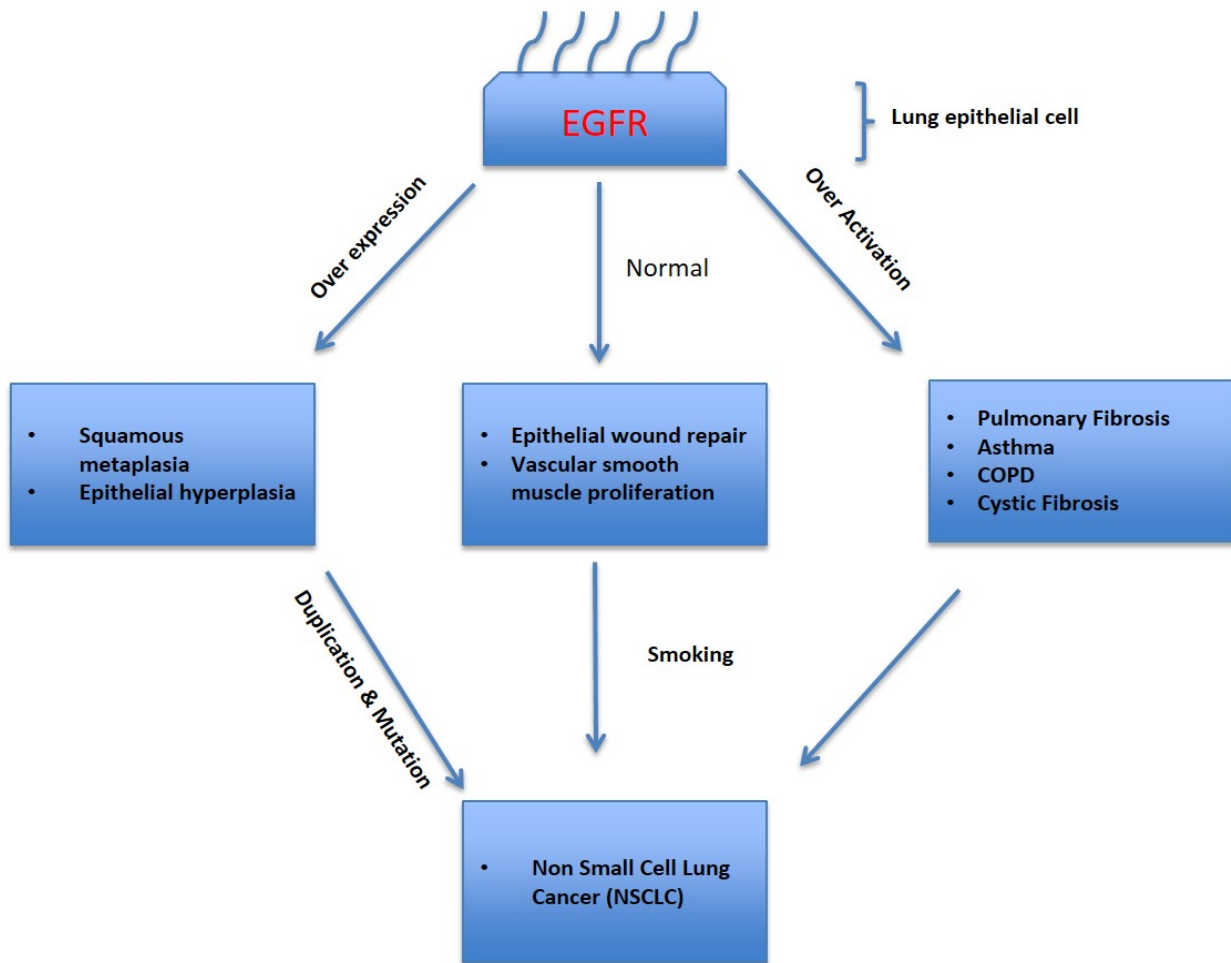


Figure 2-26: Schematic diagram of EGFR.

EGFR has been observed to work in association with EMT-related drivers and transcriptional factors. Thus, Citterio et al reported an interaction between TGF- β and EGFR assisting in cell migration and matrix turnover in EMT (Citterio & Gaillard 1994). Hui-Wen Lo et al observed EGFR mediated up regulation of STAT3-mediated (signal transducer and activator of transcription 3) Twist gene expression leading to EMT (Lo et al. 2007) and Suzuki et al demonstrated a positive correlation between an EGFR mutation and nuclear accumulation of β -catenin via synchronous alteration of Wnt and EGFR signalling pathways (Suzuki et al. 2007).

Research into EMT-activity in vivo in human smokers and COPD has also shown EGFR to be highly expressed as indicator of epithelial activation (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010) and inhaled corticosteroids reduced EGFR expression in airway epithelium in COPD in association with reducing EMT activity (Sohal, S. S. et al. 2014). Further, a recent study by Helen M et al showed that excessive expression of EGFR by airway cells may lead to squamous metaplasia in smokers with COPD (Rigden et al. 2016), possible driven by oxidative stress induce by cigarette smoke (Shaykhiev et al. 2013).

EGFR also been reported to involve in goblet cell hypertrophy and hypersecretion of mucus in smoker COPD patients, associated with increased MUC5AC gene expression but reduced MUC5B gene expression in large airways (Innes et al. 2006). It has also been reported that regulatory mechanisms governing, induction of MUC2 and MUC5AC expression was mediated by the EGFR/Ras/Raf signal transduction pathway (Perrais et al. 2002). This concept was further elaborated by Shao et al who observe that cigarette smoke activated TNF- α converting enzymes(TACE) which released mature TGF- α to bind and activate EGFR and MUC5AC in human airway epithelial cells (NCI-H292) (Shao, Nakanaga & Nadel 2004). Thus, EGFR is an integral component in COPD associated pathogenesis.

In NSCLC, the most frequent mutations found, affect K-Ras or EGFR, occurring in a mutually exclusive manner, with EGFR mutations (in exon 19 and 21) arising most prevalently in non-smokers (Pao et al. 2005), although similar somatic mutations have previously been reported in NSCLC (Lynch et al. 2004; Paez et al. 2004). EGFR over-expression leads to consequent augmentation of cell survival and proliferation has also been observed in premalignant lesions, (Hirsch, Varella-Garcia & Cappuzzo

2009). 30-75% of NSCLCs, over expressing EGFR (Soria et al. 2012) due to epigenetic changes (transcriptional hyper-activation), gene amplification or oncogenic viruses (Hirsch, Varella-Garcia & Cappuzzo 2009). It has also been reported that NSCLCs that over-express both EGFR and HER2 demonstrated an aggressive pattern of tumour cell growth. Patients bearing EGFR mutations have shown favourable clinical outcomes even with conventional chemotherapy (Eberhard et al. 2005), and is itself a therapeutic target in NSCLC (Fukuoka et al. 2003; Perez-Soler 2004).

In summary, the role of EGFR in controlling many normal cellular process including differentiation, proliferation and cell survival is very complex, and excess or aberrant expression has been implicated in tissue fibrosis, COPD and malignant transformation.

2.11.6 Epigenetic regulation of EMT

EMT has been associated with both epigenetic changes and post-transcriptional modifications of the complex processes involved.

Epigenetic modifications include those of histone protein, and DNA promoter regions, without altering DNA sequence. They include methylation of transcription promoter sites and histone modification(s) by acetylation, methylation, phosphorylation, glycosylation, hydroxylation or SUMOylation (Small Ubiquitin-like Modifier) (Kouzarides 2007). Similarly, post-translational modifications are covalent enzymatic modifications that occur during and after mRNA transcripts have been translated (Walsh, Garneau-Tsodikova & Gatto 2005).

2.11.6.A DNA methylation

This is the fundamental epigenetic modifications in mammals occurring at the 5-position of cytosine (5mC) and is catalysed by DNA methyltransferases (DNMTs) (Fukagawa et al. 2015; Subramaniam et al. 2014). The DNMT family has four members: DNMT1, DNMT3A, DNMT3B, and DNMT3L. Role of DNMT1 is to maintain DNA methylation during DNA replication, while DNMT3a and DNMT3b are associated

with de novo regulation of DNA methylation primarily during embryonic development. Hyper-methylation is associated with inactivation of CDH1 gene (E-cadherin) and is a common event in multiple human carcinomas including breast, bladder, liver gastric, prostate and lung (Bornman et al. 2001; Graff et al. 1995; Tamura et al. 2000; Yoshiura et al. 1995). Additionally, promoter hyper methylation of the CDH1 gene (E-cadherin) is positively associated with EMT in breast cancer cell lines, corresponding with an increased potential for invasion and metastasis (Lombaerts et al. 2006).

Several transcriptional factors including, Snail1, Twist and ZEB1 regulate E-cadherin expression as described previously at the level of CDH1 transcription. ZEB1 represses CDH1 transcription by its binding to two E-box sequences in the promoter. In addition, ZEB1 can also regulate CDH1 expression at the epigenetic level to induce EMT in cancer cells. DNA methylation may be of fundamental importance in the activation of pro-metastatic EMT genes (Dumont et al. 2008).

2.11.6.B Histone modifications

2.11.6.B.i Methylation

A list of histone methyltransferases involved in epigenetic regulation of EMT is given in Table 2.6, but an in depth explanation is beyond the scope of this thesis.

Snail, the zinc finger transcription factor discussed earlier, regulates EMT by suppressing the transcription of the gene for E-cadherin (CDH1) not only by binding to E-boxes in the CDH1 promoter region (Batlle et al. 2000), but also by co-operating with histone methyltransferases (HMT) and DNA methyltransferases (DNMTs) to epigenetically modulate the expression of CDH1 at this level (Lin, Y, Dong & Zhou 2014).

Hyper methylation of E-cadherin is an epigenetic modification associated with the invasive capacity of cancer cells and occurs through the interactions of EMT-TF (transcription factors) with several HMTs and DNMTs. These complexes interact to either repress the expression of CDH1, for development of a mesenchymal state, or repress the EMT-TF expression, thereby maintaining an epithelial state. Currently, several DNA methylation inhibitors are approved by the US Food and Drug

Administration (FDA). One such inhibitor, 5-Azacytidine, is well known to have good efficacy in treating myelodysplastic syndromes (Kaminskas et al. 2005; Kelly, De Carvalho & Jones 2010). The same inhibitor is also being evaluated for a blocking effect in various NSCLC cell lines, such as the anti-cisplatin cell strain A549DDP where it was observed that the methylation status of RASSF1A's (genes associated with lung cancer development and growth) was reversed by this treatment. Moreover, cell apoptosis and growth were blocked in the G0/G1 stage of cell cycle (Mengxi et al. 2013).

In general, identification of these epigenetic markers within a tumour would be helpful to develop new therapeutic approaches by targeting enzymes specifically associated with EMT in various cancers.

| HMT | Histone | Promoter |
|---------|----------|--------------|
| KDM6B | H3K27m3 | <i>SNAI1</i> |
| MMSET | H3K36m2 | <i>TWIST</i> |
| LSD1 | H3K4m2 | <i>CDH1</i> |
| Suv39H1 | H3K9m3 | <i>CDH1</i> |
| SET8 | H4K20m1 | <i>CDH1</i> |
| | | <i>CDH2</i> |
| G9a | H3K9m1/2 | <i>CDH1</i> |

Table 2.6: Key Histone Methyltransferases in EMT.

KDM6B demethylates H3K27 at the Snai1 promoter. MMSET can di or trimethylate H3K36 at the Twist promoter. LSD1 methylates H3K4m2 on CDH1 (E-cadherin gene). Suv39H1 can trimethylate H3K9 on the CDH1 promoter. SET8 methylates H4K20 on the CDH1 and CDH2 promoters. G9a is responsible for mono and dimethylation of H3K9.

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2.11.6.B.ii Acetylation

Histone acetylation and deacetylation can also play a vital role in transcription regulation (Dai & Faller 2008). Histone acetylation is catalysed by Histone Acetyl Transferases (HATs), to generate transcriptionally active chromatin. Acetylation, neutralises positively charged lysines to allow increased access to transcriptional DNA. On the other hand, histone deacetylases (HDACs) catalyse the removal of the acetyl residues to create more inactive regions (Dai & Faller 2008; Peinado et al. 2004; Tam & Weinberg 2013) (Figure 2.27). HATs and HDACs are usually part of co-activator or co-repressor multi-protein complexes, recruited to specific DNA sequences to determine the local acetylation status (Peinado et al. 2004).

Multiple HDACs have been identified in mammals and are grouped into different classes on the basis of their function and DNA sequence similarities. Class I comprises HDAC1, 2, 3, 8 and 11 whereas class II includes HDAC4, 5, 6, 7, 9 and 10. HDAC reduction observed in many chronic inflammatory diseases make it also promising therapeutic target in many fibrotic diseases (Barnes, P. J. & Stockley 2005; de Ruijter et al. 2003; Ficner 2009; Pang & Zhuang 2010). It appears that HDAC2 plays a vital role for the regulation of inflammatory genes, (Barnes, P. J. & Stockley 2005; Ito et al. 2005).

In COPD associated airway disease, a marked reduction in HDAC2 expression and activity has been observed, with rather less reduction in HDAC5 and HDAC8 expression, and normal expression of other HDACs. However, our group's reported (in rather more detailed studies) that HDAC2 expression in smokers was increased in biopsy LP (lamina propria) by smoking itself, but was reduced in current smoking COPD while ex-smoker COPD had normalised HDAC2 cell expression, there was minimal effect of inhaled corticosteroids (ICS) on this system in these tissues (Sohal, Sukhwinder Singh et al. 2013).

The majority of epithelial cancers have demonstrated over-expression of HDAC1 and HDAC2 (Jurkin et al. 2011). For example, HDAC1 was highly expressed in hepatocellular, breast, and liver, prostate, gastric and colon carcinoma; and HDAC2 was overexpressed in colorectal, cervical and gastric cancer (Bolden, Peart & Johnstone 2006; Rikimaru et al. 2007).

The role of certain HDACs is still controversial, which hinders the development of a viable therapeutic option (Adhikary et al. 2014). However, therapeutic use of HDAC inhibitors such as valproic acid (VPA), suberoylanilide hydroxamic acid (SAHA) and trichostatin-A (TSA) has already been tested in several models of cancer and showed promising results (Stadler & Allis 2012). For example, in triple-negative breast cancer, Panobinostat, an inhibitor of HDAC class I, II and IV, reduced proliferation as well as increasing the expression of E-cadherin suggesting reduced EMT activity (Tate et al. 2012). In summary, more complete understanding of histone acetylation in EMT control could well lead to more effective treatments for fibrotic and malignant conditions in the future.

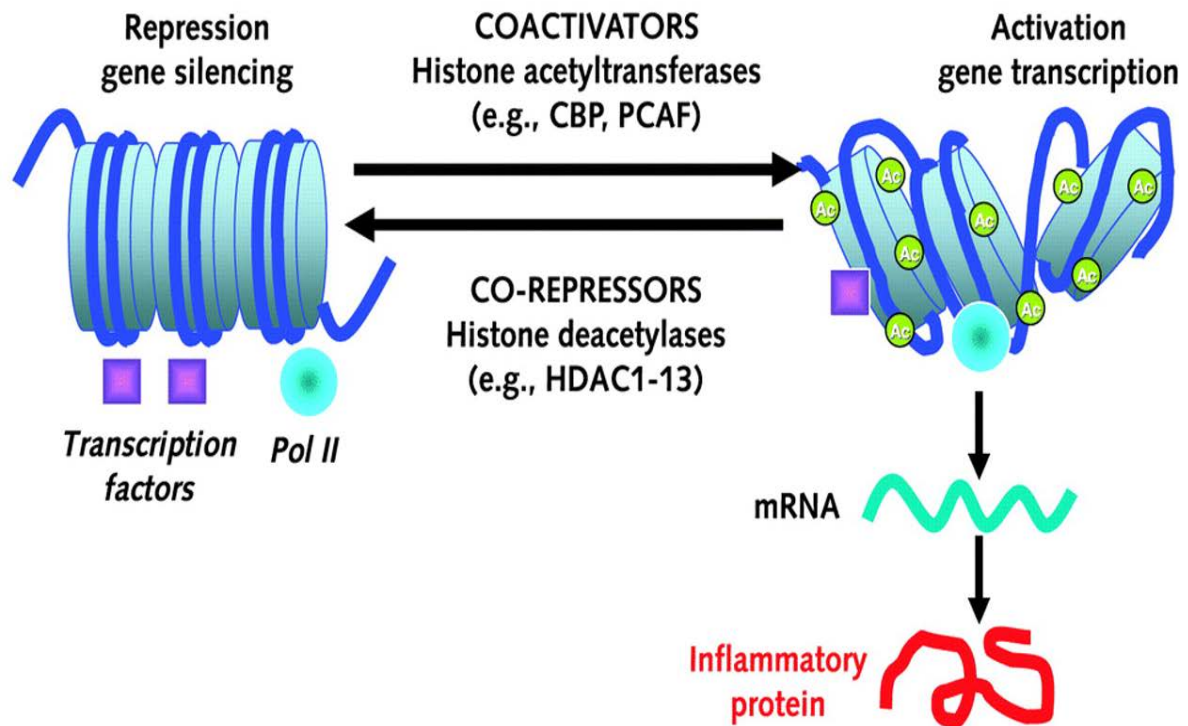


Figure 2-27: Histone acetylation.

leading to active gene transcription and deacetylation leading to gene suppression.

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2.11.6.C Post translational modifications during EMT

As mentioned, post-translational modifications (PTMs) occur covalently to proteins after mRNA translation into protein (Walsh, Garneau-Tsodikova & Gatto 2005). These covalent modifications include the addition of a modifying chemical group or another small protein addition to one or more residues of the target proteins (Deribe, Pawson & Dikic 2010). PTM can occur either on a single residue inside the protein or on multiple residues undergoing the different or same modification (Pejaver et al. 2014). An in depth literature review on PTM is beyond the scope of this thesis, but I have summarised important events in one table (Table 2.7).

| Post- translational event | Modified protein |
|---------------------------|------------------|
| Hydoxylation | HIF1A |
| Phosphorylation | SNAIL |
| | Par6 |
| SUMOylation | FoxM1 |
| | TFAP2C |
| | SIP1 |
| Glycosylation | SNAIL |

Table 2.7: Post - Translational Events in EMT.

Hydroxylated HIF-1 α (hypoxia inducible factor) promotes EMT by decreasing epithelial markers such as E-cadherin and increasing mesenchymal markers such as α -SMA and FSP1. Phosphorylation of Snail mediated by GSK-3 β results in Snail degradation by the proteasome. Snail can also be glycosylated under hyperglycaemic conditions to promote EMT.

Phosphorylated Par6 interacts with the E3-ubiquitin ligase Smurf-1 that targets RhoA for degradation, leading to the disassembly of tight junctions. The FoxM1 transcription factors belongs to a large family of forkhead box (Fox) TFs, which are characterized by the presence of a DNA-binding domain called the forkhead box or winged helix domain. SUMOylation of FoxM1 leads to repression of miR-200 EMT/tumour suppressors that enhance the expression of E-cadherin and through suppressing the expression of TFs ZEB1 and ZEB2. TFAP2C (transcription factor activator protein 2C) undergoes SUMOylation to block its ability to induce the expression of epithelial-cell luminal genes and help it to maintain basal-cell like features. SIP1 (Smad-Interacting Protein 1) SUMOylation leads to the maintenance of CDH1 (E-cadherin) expression.

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Pharmacological products, including chemical inhibitors and monoclonal antibodies, targeting several key proteins have been developed to modify disease processes at the level of EMT activity, and show promising results for the treatment of tissue fibrosis and malignancy. However, very little research has been done to target strategic post translational modifications of proteins, but would seem to have significant therapeutic potential, especially in early tumours.

2.11.7 EMT and smoking

Cigarette smoke (CigS) is recognized as an important factor for elevated risk of various respiratory diseases, as well as compromised host immunity in healthy individuals. Various ingredients, including the cigarette paper, tobacco blend, added chemicals, the type and efficiency of the filter, and the degree of tip ventilation determine the chemical composition of cigarette smoke. Thus in fresh CigS, more than 4,000 different substances have been identified so far, including neutral gases, nitrogen oxides, carbon oxides, amides, imides, lactams, carboxylic acids, esters, aldehydes, lactones, ketones, alcohols, phenols, amines, volatiles N-nitrosamines, N-heterocycles, hydrocarbons, nitriles, anhydrides, carbohydrates, ethers, nitro-compounds, metals and short- and long-living free radicals (Fowles & Dybing 2003). The quantities of the components may vary in the mainstream smoke of a single cigarette smoked from beginning to end (Borgerding & Klus 2005).

2.11.7.A Nicotine

Nicotine, a major component of cigarette smoke and the core addictive, neuro-acting agent, seems to have an important role in airway remodelling by stimulating lung fibroblast trans differentiation and upregulating Wnt signalling (Krebs et al. 2010; Rehan et al. 2005; Sakurai, R et al. 2011). Furthermore, nicotine enhances the expression of fibronectin, an EMT marker (Wei, P-L et al. 2011). Zou and colleagues observed in vitro that nicotine drive EMT in HBECs, primarily mediated via a Wnt3a/ β -catenin -dependent mechanism. They also investigated TGF- β 1 expression regulation via Wnt3a pathway, which further enhances TGF- β 1 induced EMT in HBECs (Zou et al. 2013).

This was consistent with previous studies, which had found that Wnt3a activates Wnt/ β -catenin signalling to promote EMT-like phenotypes in breast cancer cells (Wu, ZQ et al. 2012).

Nicotine is also reported to upregulate HIF1 α which has been observed to be increased in lung tissue of COPD patients and significantly related to bronchial epithelial changes and sub epithelial fibrosis (Polosukhin et al. 2007). Another important study highlighted that bronchial and alveolar epithelial cells (A549 line) underwent a phenotypic shift in response to cigarette smoke extract (CSE) exposure with increased collagen deposition again HIF1 α signalling seemed to play a role in this, implying from the above that nicotine was involved (Eurlings, IMJ et al. 2014).

An important study by Khoi et al has reported that nicotine induces Urokinase-type plasminogen activator receptor (uPAR) expression via the MAPK/ROS/NFkB signalling pathways that stimulates invasiveness in human ECV304 endothelial cells. Following this, a study by Wang and colleague, observed close association of uPA augmenting inflammation and tissue remodelling during lung injury and repair. In human small airway epithelial cells (HSAEpiCs), cigarette-smoke extract (CSE) induced uPA expression and coincident EMT, and in vivo uPA was associated with small airway remodelling in COPD patients (Wang, Q et al. 2015).

Although carcinogenic potential of nicotine is not well established, recent studies have shown that nicotine can also contribute to EMT and cell proliferation in lung cancer through the activation of nicotinic acetylcholine receptors (nAChRs) (Dasgupta et al. 2006; Dasgupta et al. 2009). Nicotine binding to nAChRs causes the recruitment of β -arrestin and Src at nicotinic receptors, with resultant activation of the MAPK pathway, and ultimately result in cell proliferation (Dave et al. 2011) (Figure 2.28). β -arrestins, a 48 kDa protein, function as scaffold proteins that form complexes with most G protein-coupled receptors (GPCRs) following agonist binding and phosphorylation of receptors by G-protein-coupled receptor kinases (GRKs) (Luttrell & Lefkowitz 2002). Src is the member of non-receptor membrane associated tyrosine kinases, which are involved in regulation of many key cellular process including cell–cell contact cytoskeletal organization, and cell–matrix adhesion. Activation of Src family kinases is observed to be associated with expression of mesenchymal proteins and other EMT related events (Liu, X & Feng 2010). Furthermore, Tsai et al observed a role for nicotine in inducing invasion and migration of NSCLC cells via $\alpha 7$ -nAChRs (Tsai &

Yang 2013). In vivo and in vitro studies of lung cancer, have shown, that nicotine could induce changes in gene expression related to EMT, characterized by decreased epithelial marker expression (E-cadherin, ZO-1) and concomitant increased acquisition and expression of mesenchymal proteins (vimentin, fibronectin), (Davis et al. 2009; Tsai & Yang 2013). In line with these studies implicating nicotine in EMT and cancer metastasis induction, Pillai et al. reported that stimulation of multiple NSCLC cell lines with nicotine led to enhanced recruitment of β -arrestin-1 and E2F1 (transcriptional regulator of cell cycle) to gene promoter region that stimulate the expression of mesenchymal markers (Pillai et al. 2015).

Periostin, also known as osteoblast-specific factor 2 (OSF-2), was observed to induce changes in tumorigenic cells (293T cells) including cell migration, invasion and adhesion (Yan, W & Shao 2006), and recently this was reinforced by demonstration of nicotine regulated periostin expression in NSCLC cells, but via nAChR-independent pathways (Wu, SQ et al. 2013).

2.11.7.B Polycyclic Aromatic Hydrocarbons (PAH)

Polycyclic Aromatic Hydrocarbons are organic compounds released as a result of incomplete combustion of coal, gas or tobacco. The role of major PAH in EMT induction was first noticed when Benzo-a-pyrene, was observed to induce activation of EMT-related genes including TGF β 2, Twist, plasminogen activator inhibitor-1, fibronectin, and basic fibroblast growth factor in A549 cells (Yoshino et al. 2007). In addition, PAH also activate a ligand-activated transcription factor (aryl hydrocarbon receptor-AhR) which was observed to participate in Slug induction, and then EMT (Ikuta & Kawajiri 2006) (Figure 2.28). AhR expression was observed to be upregulated in lung adenocarcinoma, suggesting a presumptive role in its development and progression (Lin, P et al.). Valavanidis et al reported that in a malignant setting, marked cytoskeleton remodelling is indeed the result of prolonged AhR activation and resulting JNK pathway activation which ultimately leads to increased cell migration and invasion (Valavanidis, Vlachogianni & Fiotakis 2009). An essential role of AhR in EMT was also evidenced by CSE inducing MMP-1 expression in human fibroblasts through activation of the AhR-related pathway (Ono et al. 2013).

2.11.7.C Reactive Oxygen Species (ROS)

CigS also contains reactive oxygen species (ROS), which are stable and unstable free radicals in the particulate and the gas phases that can lead to oxidative damage to lung tissue (Valavanidis, Vlachogianni & Fiotakis 2009). Although the carcinogenic role of oxidative damage by cigarette smoke is unclear (Pfeifer et al. 2002) it has been observed that hydroxyl radicals generated from cigarette tar can cause oxidative DNA damage. In addition, CigS and respirable fibres and dusts act synergistically to produce increase amount of damaging hydroxyl radicals (Valavanidis, Vlachogianni & Fiotakis 2009). Studies have also shown that various oxidants present in CigS including peroxynitrite and H₂O₂ (Mallozzi, Di Stasi & Minetti 1999; Mehdi et al. 2005) can activate Src in a NSCLC cell line (H358), resulting in EMT-associated cell invasion and motility (Zhang, H et al. 2012) (Fig 2.28).

2.11.7.D Nicotine-derived nitrosamine ketone (NNK)

NNK has been found to upregulate the expression of the mesenchymal marker fibronectin through cyclooxygenase-2 (COX-2), which is mediated by the activation of $\alpha 7$ -nAChR. Polycyclic aromatic hydrocarbons (PAH) can mediate EMT through the activation of the aryl hydrocarbon receptor (AhR), which triggers marked cytoskeleton remodelling associated with the activation of the JNK pathway. Increased nuclear accumulation of AhR in response to PAH also leads to transcriptional activation of Slug (Fig 2.28).

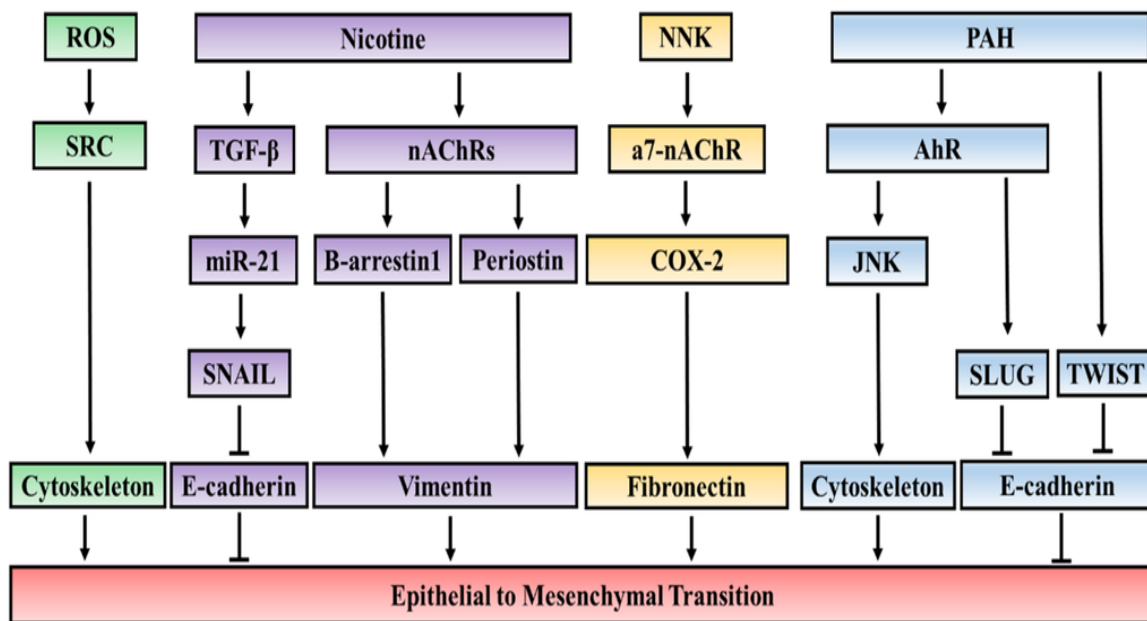


Figure 2-28: EMT and smoking: Summary

Reactive oxygen species (ROS) found in cigarette smoke can activate SRC resulting in cytoskeletal modification. Nicotine can induce EMT through nAChRs-dependent and nAChRs-independent pathways. In the nAChRs-dependent pathway, the activation of nAChRs results in the recruitment of β -arrestin1 on to the promoters and promotes the expression of mesenchymal markers. Additionally, the activation of nAChRs can also induce expression of periostin, the overexpression of which has been reported to cause EMT. In a nAChRs-independent pathway, nicotine can induce EMT through upregulating miR-21 in a manner dependent on TGF- β signalling.

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2.11.8 EMT and Angiogenesis

The role of new blood vessel formation in EMT has not been widely discussed in the literature. However, angiogenesis has been studied as a vital aspect of both premalignant and malignant phases of cancer progression (Hanahan & Weinberg 2011). Previous studies have demonstrated that smokers with neo-angiogenesis and projection of capillary loops penetrating into the bronchial epithelium are at especially high risk of developing lung cancer (Hiroshima, K. et al. 2002; Keith et al. 2000).

Studies by our research group have reported increased vascularity in the sub-epithelial reticular basement membrane (Rbm) of the bronchial mucosa in COPD subjects (Soltani, A. et al. 2010; Soltani, Wood-Baker, et al. 2012). These aberrant vessels were found to be hyper permeable using surrounding albumin as a marker of vessel leakage. This increased vessel leakiness is consistent with previous literature describing vessels associated with premalignant lesions (Hanahan & Weinberg 2011; Soltani, A. et al. 2010; Soltani, Wood-Baker, et al. 2012).

A subsequent study by Soltani et al investigated TGF- β expressing blood vessels, providing evidence for involvement of this major signalling molecule in this angiogenesis (Soltani, Sohal, et al. 2012). These TGF- β -positive vessels were detected in the Rbm of smokers and COPD subjects with evidence of EMT (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010, 2011). All these findings are consistent with active EMT Type 3, and previous literature showing potential of various EMT markers including S100A4 to be involved in the induction of angiogenesis (Kalluri, R. & Neilson, E. G. 2003).

Endothelial cells of tumour associated blood vessels have been found to express endocan in non-small cell lung cancer (NSCLC) (Grigoriu et al. 2006). Endocan has been found to inhibit leukocyte adhesion to endothelial intercellular adhesion molecule-1, (ICAM-1), and lymphocyte function-associated antigen-1, (LFA-1) and their subsequent migration into tissue (Caires et al. 2009; Sarrazin et al. 2010). In particular, endocan also has the potential to inhibit migration of Natural Killer (NK) cells which should play an important role in recognising and killing of tumour cells (Sarrazin et al. 2010). This may link vessel proliferation in COPD airways, i.e. Type 3 EMT and increased risk of lung cancer.

Fibroblast-like cells produced through EMT are also believed to be involved in modulating cancer progression (Kalluri, Raghu & Zeisberg 2006). Thus, it has been postulated that EMT may give rise to cancer-stimulating stromal cells (cancer associated fibroblasts, CAFs) which produce tumour enhancing growth factors (Iwatsuki et al. 2010).

2.11.9 EMT in COPD

As described previously, COPD is characterized by the poorly reversible airflow obstruction, mainly due to inhalation of cigarette smoke (Churg et al. 2006; Hogg, Macklem & Thurlbeck 1968; Saetta, M. et al. 2000). Fibrosis and obliteration of the small airways and in some individual's later development of emphysema contribute to this progressive airflow obstruction (O'Donnell, DE & Parker 2006).

It has recently been shown that a fundamental-level pathology in COPD is genetic reprogramming of epithelial basal stem cells, consequence of which is a markedly altered epithelium structure; EMT induction may also be part of this process. However, details of the processes involved are not yet clearly understood.

A study by our group a decade ago was the first to directly implicate EMT in COPD. This study showed that in addition to the classic changes of squamous metaplasia and (epithelial thickening), goblet cell and mucous gland hyperplasia, there is also marked fragmentation of the Rbm and mesenchymal protein acquisition by basal epithelial transitioning cells in smokers but especially in currently smoking COPD (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010). In addition, large numbers of EMT-associated and presumably transitioning cells within clefts in the Rbm show increase expression of MMP9 and S100A4. The fragmentation of the Rbm is likely due to the MMP digesting the way for the cells through to the lamina propria (Acloque et al. 2009; Kalluri, R. 2009; Kalluri, R. & Neilson, E. G. 2003; Zeisberg & Neilson 2009). The cells in the fragmented Rbm were shown to double-express both epithelial and mesenchymal markers (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010, 2011). All of these observations were made using large airway endobronchial biopsies taken bronchoscopically from phenotypically diverse research volunteers. In EMT, activated state of large airway epithelium was observed, evident with overexpression

of EGFR (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010). As previously discussed, the group also observed hyper-vascularity of Rbm and epithelium and hypo-vascularity of the LP, but with this change only found in current smokers, with or without COPD (Soltani, A. et al. 2010).

Subsequently, Milara et al demonstrated that cigarette smoke extract (CSE) was capable of inducing EMT in vitro primary human bronchial epithelial cells through the TGF β pathway but antagonised by increased cAMP levels (Milara, Peiro, et al. 2013). Similarly, Wang et al. contributed by observing a significant correlation between uPAR and vimentin (a mesenchymal marker) expression in COPD airway epithelium and in association with CSE-induced EMT in-vitro (Wang, Q et al. 2013). Later Sophie and colleagues highlighted the importance of EMT by describing that bronchial epithelium from COPD patients showed increased vimentin and decreased ZO-1 and E-cadherin expression. At the same time vimentin over-expression was also significantly correlated with basement membrane thickening and (importantly) airflow limitation. In addition, EMT-related de-differentiation of the epithelium occur in COPD conducting airways and correlated with peri-bronchial fibrosis and with airflow limitation. They also recapitulated these findings in vitro, where re-differentiation of the epithelium seemed to be the consequence of TGF- β signalling. Moreover, targeting TGF- β 1 during in vitro differentiation also prevented vimentin induction and fibronectin release (Gohy, S. T. et al. 2015).

2.11.10 EMT in Lung Cancer

Lung cancer is a major cause of death worldwide (Siegel, R., Naishadham, D. & Jemal, A. 2013), predominantly in smokers and especially in those with COPD. It is highly complex neoplasm with several histologic types, with primary division between small cell and non-small cell lung carcinoma (NSCLC), with adenocarcinoma and squamous carcinoma being the predominant types in NSCLC (Molina et al. 2008). Lung cancer are believed to result from progressive series of cellular events (Wistuba 2007) that also involve EMT. A deeper understanding of mechanism involved in EMT associated with lung cancer progression and metastasis may lead to new and effective strategies for early detection and chemoprevention and targeted treatment.

FSP-1(S100A4) is a “classic” mesenchymal protein marker in which activity corresponds to fibroblast-regulated fibrosis in pulmonary tissue (Liau, Yamada & de Crombrughe 1985). This might also give a clue to EMT associated fibroblast/myofibroblast, expressing mesenchymal protein in malignant stroma. It is interesting to note that other sources of myofibroblasts in tumour tissue include pericytes, resident fibroblasts and bone-marrow derived mesenchymal stem cells. (Navab et al. 2011; Venkov et al. 2007). Further, gene expression of proteins regulated by TGF β and MAPK signaling were observed to be higher in cancer-associated fibroblasts (CAF) in comparison to normal fibroblasts (Navab et al. 2011). Shi et al observed that EMT associated cancer cells, promote expansion of mesenchymal cells and poor patient outcomes correlated with elevated Twist, Snail and CD44 expression in lung cancer (Shi et al. 2013).

Walser and colleagues proposed EMT as an important linking feature between COPD and lung cancer where it was highlighted that the cytokines and growth factors aberrantly produced in COPD and the developing tumour microenvironment have been found to have deleterious properties that simultaneously pave the way for both EMT and destruction of specific host cell-mediated immune responses (Walser et al. 2008). EMT in the context of lung cancer, has been reported to employ various signalling mechanisms and down-stream transcriptional factors for development, progression and finally metastasis. Here I will give a brief review of each for lung cancer, trying not to repeat too much of my previous discussion (Fig 2.29). It has been shown that EMT requires the cooperation of oncogenic Ras or RTK, both of which induce hyperactive downstream signaling of Raf/MAPK with endogenous TGF β signaling. Indeed, it has been observed that sustained TGF β signaling may be required for the maintenance of EMT in epithelial cells and also for metastasis in several mouse models (Grunert, Jechlinger & Beug 2003; Morris et al. 2003); TGF β_2 may be particularly important in lung cancers, both NSCLC and SCLC (Malkoski et al. 2012) and may be a potential therapeutic target (Fong et al. ; Tang et al. 2006).

Wnt signaling has also been demonstrated in many human cancers and cells expressing Wnt-1 are resistant to therapies that mediate apoptosis. Thus, Wnt-1 and Wnt-2 have been found to be over-expressed in NSCLC lines (Huang, C et al. 2015). In addition, anti-Wnt-1, a monoclonal antibody, has been shown to suppress tumor growth (You et al. 2004).

We know that lung cancer (SCLC and NSCLC) requires an active Hh signaling pathway. It has been shown that Hh pathway antagonists also inhibit proliferation of NSCLC cell lines (Yuan et al. 2006). The importance of Hh signaling in cancer lies in the transcription targets of Gli, and other genes known to control cell proliferation: i.e, cyclin D1, cyclin E1 and Myc. Gli is also reported to activate genes controlling tumor angiogenesis including those in VEGF and PDGF signaling pathways (Morrow et al. 2009). Endothelial cells lining the blood vessels supplying the tumor with nutrients also demonstrate endo-MT, under the influence of PDGF, periostin and Hh signaling pathways (Smith & Bhowmick 2016).

Notch signaling (Notch receptor and ligand) were shown to be elevated in NSCLC lines and blockade of Notch pathway activation, using gamma secretase inhibitor caused increased apoptosis and reduced NSCLC tumor growth (Konishi et al. 2007). More recently, it has been shown that M2-like tumor-associated macrophages (TAMs) have a lower level of Notch pathway activation in mouse tumor models, while forced activation of Notch signaling in macrophages enhanced their anti-tumor capacity (Wang, YC et al. 2010). Therefore, Notch signaling plays critical roles in the determination of M1 versus M2 polarization of macrophages, and compromised Notch pathway activation can lead to over-expression of the M2-like TAMs in lung cancer progression (Popper 2016).

Regarding therapeutic potentials for COPD, extensive review by Young and colleagues have highlighted the potential for statins in COPD. Current therapies for COPD have been shown to reduce symptoms and infective exacerbations and to improve quality of life. However, these drugs have little effect on the natural history of the disease (progressive decline in lung function and exercise tolerance) and do not improve mortality. The anti-inflammatory effects of statins on both pulmonary and systemic inflammation through inhibition of guanosine triphosphatase and NFkB-mediated activation of inflammatory and matrix remodeling pathways could have substantial benefits in patients with COPD due to the following: 1) Inhibition of cytokine production (tumor necrosis factor- α , interleukin (IL)-6 and IL-8) and neutrophil infiltration into the lung; 2) inhibition of fibrotic activity in the lung leading to small airways narrowing and irreversible airflow limitation; 3) antioxidant and anti-inflammatory (IL-6 mediated) effects on skeletal muscle; 4) reduced inflammatory

responses to pulmonary infection; and 5) inhibition of the development (or reversal) of EMT, a “precursor event to lung cancer” (Young, Hopkins & Eaton 2009).

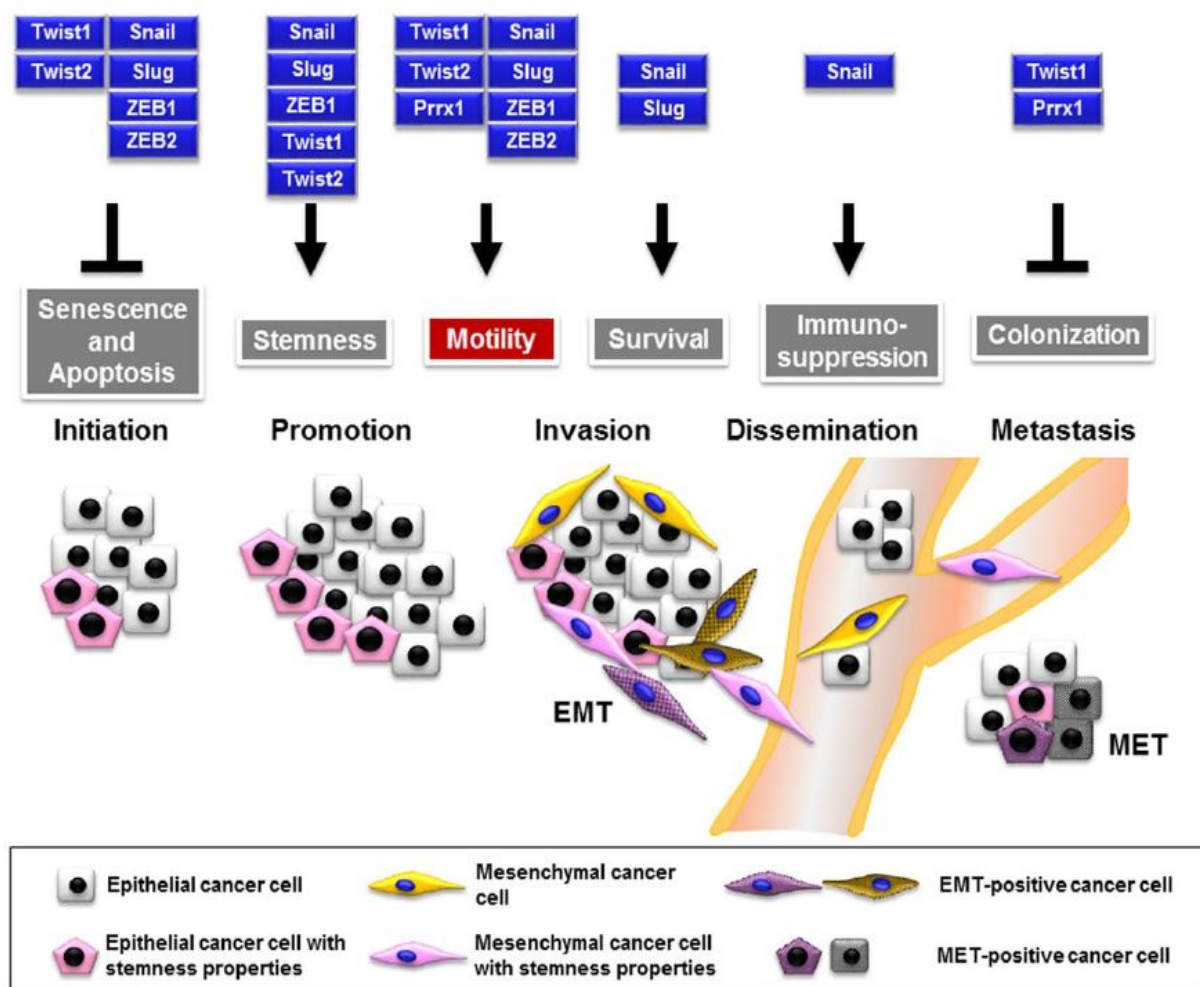


Figure 2-29: Multiple steps in Cancer Progression.

Involvement of EMT-inducing transcription factors (EMT-TFs).

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There is emerging evidence of cancer stem cell (CSC) involved in development of malignancy including lung cancer, which is currently under active investigation. The CSC model is based on the fact that each tumour contains a small subpopulation of cells which are capable of both cancer self-renewal and to generating differentiated, non-tumorigenic daughter cells (Clarke et al. 2006). In the lung, bronchio-alveolar stem cells (BASCs) are a population of pulmonary stem cells which are actually progenitors of airway epithelial (these stem cells are the epithelial basal cells) and alveolar type I and type II cells. They are crucial for renewal of bronchial, bronchiolar and alveolar

epithelium (Kim, CF 2007) and that their transformation may give rise to lung adenocarcinomas (Kim, CFB et al.). This supports a previous mouse model study where it was observed that BASC numbers were increased, even in the earliest malignant lesions that further increased during tumor progression (Jackson et al. 2001). The ability of EMT markers to identify a subset of tumour cells raises the possibility that EMT could be associated with the maintenance of cancer stem cells. Thus Brabletz reported that invading cells expressed nuclear β -catenin as well as the stem cell markers hTert and survivin (Brabletz, Jung, et al. 2005).

The role of EMT in tumour metastasis has been widely studied. The presence of EMT markers at the tumour–host interface, but not in the central bulk of tumour, is strong evidence that EMT is involved in tumour invasiveness and aggressiveness. Two hypotheses currently attempt to explain EMT and metastasis 1) cancer progenitor cells initially undergo EMT and then metastasis following clonal expansion. This would cause a metastatic tumour to share a signature with the cell that originally underwent EMT, and thus influences cancer grade (Sarkar, S et al. 2013). 2). It has been also observed that, for successful metastasis there is another process, mesenchymal-epithelial transition (MET). Cancer cells undergone EMT, travelled to other parts of the body usually have a mechanism that aid them to infiltrate in other tissues and produce new, clinically significant tumour sites, possibly by induced pluripotent stem cells (iPSCs) which regain epithelial characteristics and so anchor themselves in the normal surrounding tissue (Polo & Hochedlinger 2010).

2.12 Concluding remarks for literature review and questions for the present thesis

There is obviously increasing interest in the role of EMT in the pathogenesis of the airway wall fibrosis that underlies COPD, as well as its association with development and metastasis of lung cancer. Indeed, the majority (50–60%) of lung carcinoma arise in the setting of smoking related airway disease i.e. there is a strong link between lung cancer and airflow obstruction, suggesting that there may be common underlying pathogenic mechanisms. Notably, two distinct types of EMT have been described in relation to fibrosis in general and malignant transformation. In addition, EMT drivers, pathways and downstream transcription factors have been implicated as major etiological factors in the pathogenesis in COPD. Despite all the data, the mechanisms

by which EMT is driving this process in airways of COPD have still been are poorly understood and importantly still not generally accepted in the respiratory research or clinical communities where an inflammatory model of COPD pathogenesis still holds major sway.

The first step in the EMT process in the context of disease process is the “activation” of airway epithelial cells, however that is defined, that leads to structural alteration in epithelial morphology, and the characteristics gaining of mesenchymal potential. Several specific questions arise regarding mechanisms: Firstly, which specific subtype of EMT is present in both small and large airway of smokers and how much EMT activity related to airflow obstruction. Secondly, what transcriptional pathways (excitatory and inhibitory) are in play and how do they correlate with the degree of EMT activity in both smokers and COPD patients and how do they relate to airflow obstruction. Thirdly, whether EMT has role in, development, progression and metastasis of NSCLC in smokers and how does this relate to EMT in the airways of origin.

Thus, the **aims** of my thesis were developed to answer these key questions in the context of the early airway disease before confounding by chronic infection and emphysema are prevalent; and are summarized below;

1. To assess epithelial activation and EMT type and activity in small airways compared to matching large airways, I wish to evaluate the relationship between epithelial activation and EMT activity and the degree of airflow obstruction.
2. To assess TGF β 1-pSmad 2/3 upregulation and whether there was reciprocal down regulation of inhibitory pSmad7 in the epithelium, Rbm and LP of large airways in smokers and COPD patients, I choose large airways because I found that EMT activity was greatest there, and because I had access to abundant tissue from very well characterized, age-matched volunteer subject groups, I also evaluated the relationship of expression of this pathway to the degree of airflow obstruction and EMT activity.
3. To assess not only β -catenin/Snail1/Twist TF expression but also their cellular localization, again in large airways of smokers and COPD patients. Once more,

I also evaluated the relationship of these TFs to the TGF β -Smad pathway, degree of airflow obstruction and EMT activity.

4. To compare the expression pattern of EMT biomarker between central and peripheral (leading edge) areas of NSCLC, evaluate the relationship between EMT biomarker expression with differentiation and staging of NSCLC and also to evaluate the relationship of the EMT expression and vascularity in tumor with changes in the corresponding small and large airway wall.

2.12.1 Hypothesis

The overall hypothesis for my PhD studies is that EMT is an important pathogenic mechanism in the development of COPD. I have taken this further by hypothesising in addition, that well-accepted core EMT-related pathways and TFs can be demonstrated as active in the airways of COPD patients, and are related to functional manifestations of disease; and finally, that EMT is a process that relates COPD to its major complication of lung cancer development and its early dissemination.

Chapter 3

3. Materials and Methods

3.1 Introduction

For the *ex-vivo* study of expression of epithelial activation and EMT bio-markers, I used: firstly, resected lung material to be able to compare small and large airways, while secondly, for investigation of pathways involved in EMT activation, I used only large airway biopsies obtained bronchoscopically from volunteers, including normal non-smokers. In both groups, we carefully phenotyped individuals has being current or ex-smokers, and having normal spirometry, or small airway obstruction only, or frank COPD i.e. with an FEV1/FVC ratio of < 70%. I was able to obtain lung material from age-matched deceased individuals from a lung bio-bank at the University of British Columbia in Canada, through the kind auspices of Prof. Jim Hogg (Its Director) and Prof Darryl Knight, at that time visiting fellow.

Throughout, I have studied protein expression using immune-staining: in Chapter 4 and 7, for bio-markers of epithelial mesenchymal transition (EMT), S100A4 and vimentin; EGFR as an epithelial cell activation marker and as a marker of blood vessels, Type-IV collagen. In the same way in Chapter 5 and 6, I stained for expression of TGFβ1/pSmad 2/3, pSmad 7, β-catenin, Snail1 and Twist.

3.2 Subject recruitment

3.2.1 Ethics statements

The Tasmania Health & Medical Human Research Ethics Committee approved all studies (EC00337, H0013051 and H0012921). All subjects gave written, informed consent to use their tissue, either prior to volunteer bronchoscopy and biopsy, or prior to lung surgery.

3.2.2 Endo-bronchial biopsies from large airways

Twenty current smokers with established COPD (COPD-CS), 15 current smokers with normal lung function (NLFS), 15 ex-smokers with COPD (COPD-ES) and 15 normal healthy, never-smoking controls (NC) were recruited by advertisement in local newspapers and placement of posters in clinic waiting areas in the Royal Hobart hospital, as well as on the notice boards of social and veterans clubs. Potential participants were interviewed and examined by a respiratory physician clinically and physiologically. The diagnosis of COPD was made according to GOLD guidelines based on FEV₁/FVC ratio and categorized into two groups on the basis of current versus ex-smoking history. Data on this group are provided in corresponding Chapter 5 and 6.

For normal lung function current smokers, the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits, i.e., FEV₁ >80% of predicted, and FERatio (FEV₁/FVC) >70% and no significant scalloping out of the expiratory descending limb of the flow-volume curve to suggest small airway obstruction. Normal healthy volunteers had normal lung function by spirometer assessment and had no history of smoking and respiratory illness.

3.2.3 Exclusion criteria

1. Subjects with a history of asthma, which included symptoms in childhood, related significant atopic disorders, eczema or hay fever, substantial day-to-day variability or prominent nocturnal symptoms, or a history of wheeze rather than progressive breathlessness and any who had previously used ICS (oral or inhaled) were excluded.
2. Significant uncontrolled comorbidities such as diabetes, angina or cardiac failure, and other coexisting respiratory disorders including pulmonary fibrosis, lung cancer and bronchiectasis.
3. Subjects with inability to give written informed consent were also excluded.

3.2.4 Inclusion criteria for phenotypes

1. Current-smokers with COPD aged at least 40 years with smoking history equal to or more than 15 pack-years; FEV₁ 40% to 80% predicted, with FER (FEV₁/FVC) ≤70% post bronchodilator with definite scalloping out of the

descending limb of flow-volume loop on spirometry. COPD ex-smokers with at least six months of smoking cessation were included.

2. Normal healthy never smoking controls and current smokers with normal lung function recruited also underwent bronchoscopic examination and physiological evaluation. They were at least 18 years old and FEV₁/FVC ratio of 70% or higher and FEV₁% predicted of 80% or higher.
3. None of the never-smoking controls individuals had any history of respiratory illness or smoking. For normal lung function current smokers, the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits (FEV₁ >80% of predicted, and FERatio (FEV₁/FVC) >70%) and no scalloping out of the expiratory descending limb of the flow-volume curve, suggesting small airway obstruction.
4. Individuals with Small airway disease (SAD) recruited on the basis of significant scalloping of the expiratory limb of the flow-volume curve and FEF₂₅₋₇₅ < 68% predicted.

3.3 Bronchoscopy

Standard techniques were used in performing fiberoptic bronchoscopy. Subjects were pre-medicated with nebulized salbutamol (5 mg) 15–30 min before the procedure. Intravenous midazolam (3–10 mg) and fentanyl (25–100 µg) was used for sedation. Topical anesthesia above the vocal cords was done by lignocaine (4%) and 2% lignocaine was used to anaesthetize the airways below the cords, in 2 ml aliquots as required, up to a maximum of 6 ml. Subjects were monitored by pulse oximetry throughout the procedure and oxygen was administered routinely. Biopsies from secondary carina of segmental and sub-segmental bronchi in the right lower lobe were obtained, which were eight in number. There were no complications from the procedures. Bronchial biopsies were fixed in 4% neutral buffered formalin for 2 hours and subsequently processed into paraffin through graded alcohol and xylene using a Leica ASP 200 tissue processor.

3.4 Resected lung tissue non-tumorous large and small airways, and also lung cancers

Twenty-five patients were included having primary non-small cell lung cancer (NSCLC), with an approximately equal distribution of squamous and adenocarcinoma. Patients were classified as current smokers or ex-smokers (at least 12 months of smoking cessation). Nine patients had demonstrated GOLD stage I/II COPD on post-bronchodilator spirometry ($FEV_1 < 70\%$), and Nine patients had small airway disease (SAD) only, with scalloping of the expiratory limb of the flow-volume curve and $FEF_{25-75} < 68\%$ predicted (Ciprandi et al. 2012). In addition, there were seven individuals who were current smokers without evidence of airflow obstruction, and hence designated as normal lung function smoker (NLFS). Again, those with a history of other chronic respiratory disorders including clinico-pathological asthma were excluded. Surgical resection material containing non-cancerous small and large airways and away from the main tumor, were fixed in formalin immediately after the surgery. At processing, tissue blocks of small airway (< 2.0 mm internal diameter) were embedded in paraffin for our analyses.

For the study comparing EMT expression in small versus large airways, I merged the COPD and those subjects who had only SAD, under the heading of CAL (chronic airflow limitations) because in fact there was essentially no difference between them in EMT bio-marker expression. Details are given in relevant chapter 4.

Nine non-smoking, non-COPD subjects were included in the large versus small airway comparison study, as a control group (NC). As mention above, I am thankful to Prof. Darryl Knight (University of Newcastle, Australia) and Prof. J.C. Hogg (University of British Columbia, Canada) for assistance in providing normal small airway tissues. I am also thankful to Professor HK Muller, a professional pathologist, who inspected the tissue microscopically and confirmed that these tissues were normal, and without any evidence of smoking or changes associated with chronic respiratory disease.

For NSCLC study, the details of patient included with adeno and squamous cell-carcinoma are given in Chapter 7.

3.5 Processing of biopsies and resected tissue

Tissue acquisition was from the Royal Hobart Hospital (RHH), but tissue grossing for proper identification was all done by me in RHH Pathology department. Regarding optimization of immunostaining and finalized staining work, I closely work (30-40%) with Mr. Steve Weston, our highly experienced lab manager. In addition, for quality control purpose, Steve oversaw all my processing work, while the more routine work of cutting sections and staining was done by Steve, at least for the most part when he was available. Sections cut at 3 μ m intervals from individual paraffin blocks were stained with hematoxylin & eosin and assessed for quality and lack of damage. Following removal of paraffin and rehydration, immunostaining for EGFR, S100A4, Vimentin, N-cadherin, Type-IV collagen, TGF β 1, pSmad 2/3, pSmad7, β -catenin, Snail1 and Twist was done as appropriate in the different studies. For each individual study (Chapter 4-7), antibody staining was performed in 2 batches because the numbers of sections needing processing by the autostainer was too great for its capacity. However, appropriate verification controls were run with each batch, with coding for specific phenotypes done independently and blind to me. During staining optimization, we choose an antibody dilution and image analyzer setting that gave an optimal output. We kept to these setting throughout subsequent analysis and changes nothing from slide to slide or day to day.

3.5.1 General procedure for hematoxylin and eosin (H&E) stain

1. Paraffin blocks were sectioned at 3 μ ms after cooling in -20°C freezer or on ice blocks 5-10 minutes.
2. For biopsies, two sections (approximately 3 μ m thick) separated by 40-50 micron (approximately 10 sections) were cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). For resected tissue, only one tissue section was used on glass slide because of large size of resected tissue.
3. Slides were dried at 56°C for one hour and overnight at 37°C .
4. In order to elaborate nuclei, slides with tissue sections were placed in Mayer's hematoxylin for 5minutes, followed by rinsing in running water.

5. Tissue sections were placed in approximately 400ml of water with 8 drops of ammonia 30 seconds, and rinsed well in running water.
6. Slides were then placed in eosin solution for 2 minutes, and quickly rinsed in running water to remove excess eosin and then placed into 95% ethanol for 30 seconds.
7. Further Dehydration of sections were done in clean fresh 95% ethanol, and then changed twice with 100% ethanol (2 minutes each change).
8. Clearing of sections was done using two changes of fresh xylene (2 minutes each).
9. Sections were coverslipped by using Dako Coverslipper (Dako, Denmark A/S) with 54 mm coverslipped and dried on hotplate overnight.

3.5.2 General procedure for EGFR staining

1. Paraffin blocks were sectioned at 3µms after cooling in –20 freezer or on ice blocks 5-10 minutes.
2. One section (approximately 3µm thick) of tissue was cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
3. Slides were labelled using printed labels and marked on the edges with wax pen.
4. Dewaxing of sections was done in “xylene” (twice for 5 minutes each) in fume hood.
5. Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections well in running tap water (2 minutes).
6. Antigen retrieval was performed for EGFR; sections were kept in proteinase K solution for 5 minutes.
7. After antigen retrieval, the slides were cooled down to room temperature and transferred to Dako Autostainer Plus (Dako, Denmark, A/S), in which a program template for staining was made.

8. For EGFR, sections were placed in 3% H₂O₂ in distilled water for 20 minutes and washed in distilled water for 2 minutes to block endogenous peroxidase activity.
9. Sections were rinsed in working Tris HCl buffer for 2 minutes after each treatment.
10. Primary antibody was applied to the sections; EGFR (Clone H11, Dako, Denmark, Catalogue no. M3563, 1/100 dilution for 90 minutes) A negative control was also used in each run with isotyped matched immunoglobulin IgG1 (clone X0931 Dako Denmark A/S). Lung sections from previous biobank were used as positive controls for confirmation of staining.
11. Sections were rinsed well using Tris HCl (thrice, 5 mins each).
12. Applied EnVision+ system-HRP labelled polymer (catalogue number K4001; Dako, Denmark A/S) reagent to sections for 30 mins and rinsed with Tris HCl buffer.
13. DAB+ (catalogue number K3468; Dako, Denmark A/S) was applied to sections for 10 minutes and rinsed well using Tris HCl (twice) followed by distilled water.
14. The slides were taken out of Autostainer and put in Mayers hematoxylin to elaborate nuclei for 3-5 minutes, then rinsed in running water.
15. Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds, and rinsed well in running water.
16. Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each).
17. Clearing was done in two changes of xylene (2 minutes each).
18. Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.5.3 General procedure for S100A4 and Vimentin staining

- 1 Paraffin blocks were sectioned at 3µms after cooling in -20 freezer or on ice blocks 5-10 minutes.
- 2 One section (approximately 3µm thick) of tissue was cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark

- A/S). Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
- 3 Slides were labelled using printed labels and marked on the edges with wax pen.
 - 4 Dewaxing of sections was done in “xylene” (twice for 5 minutes each) in fume hood.
 - 5 Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections well in running tap water (2 minutes).
 - 6 Antigen retrieval was performed in PT Link (Dako, Denmark A/S): for S100A4, sections were kept at 95°C at high pH buffer for 20 minutes: for vimentin, a temperature of 97°C at high pH buffer for 15 minutes was used.
 - 7 After antigen retrieval, the slides were cooled down to room temperature and transferred to Dako Autostainer Plus (Dako, Denmark, A/S), in which a program template for staining was made.
 - 8 For both S100A4 and vimentin, sections were placed in 3% H₂O₂ in distilled water for 15 minutes and washed in distilled water for 2 minutes to block endogenous peroxidase activity.
 - 9 Sections were rinsed in working Tris HCl buffer for 2 minutes after each treatment.
 - 10 Primary antibody was applied to the sections; S100A4 (Dako, Denmark, Catalogue no. A5114, 1/2000 dilution for 90 minutes) and vimentin (Clone vim3B4 Dako, Denmark, Catalogue no. M7020, 1/1000 for 60 minutes). A negative control was also used in each run with isotyped matched immunoglobulin IgG1 (clone X0931 Dako Denmark A/S). Lung sections from previous biobank were used as positive controls for confirmation of staining.
 - 11 Sections were rinsed well using Tris HCl (thrice, 5 mins each).
 - 12 Applied EnVision+ system-HRP labelled polymer (catalogue number K4001; Dako, Denmark A/S) reagent to sections for 30 mins and rinsed with Tris HCl buffer.
 - 13 DAB+ (catalogue number K3468; Dako, Denmark A/S) was applied to sections for 10 minutes and rinsed well using Tris HCl (twice) followed by distilled water.

- 14 The slides were taken out of Autostainer and put in Mayers hematoxylin to elaborate nuclei for 3-5 minutes, then rinsed in running water.
- 15 Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds, and rinsed well in running water.
- 16 Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each).
- 17 Clearing was done in two changes of xylene (2 minutes each).
- 18 Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.5.4 General procedure for N-cadherin and Type-IV collagen staining.

- 1 Paraffin blocks were sectioned at 3µms after cooling in –20 freezer or on ice blocks 5-10 minutes.
- 2 One section (approximately 3µm thick) of tissue was cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approximately 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
- 3 Slides were labelled using printed labels and marked on the edges with wax pen.
- 4 Dewaxing of sections was done in “xylene” (twice for 5 minutes each) in fume hood.
- 5 Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections well in running tap water (2 minutes).
- 6 Antigen retrieval was performed in PT Link (Dako, Denmark A/S): for N-cadherin, sections were kept at 97°C at high pH buffer for 30 minutes: for Type-IV collagen, a temperature of 97°C at high pH buffer for 20 minutes was used.
- 7 After antigen retrieval, the slides were cooled down to room temperature and transferred to Dako Autostainer Plus (Dako, Denmark, A/S), in which a program template for staining was made.

- 8 For both N-cadherin and Type-IV collagen, sections were placed in 3% H₂O₂ in distilled water for 15 minutes and washed in distilled water for 2 minutes to block endogenous peroxidase activity.
- 9 Sections were rinsed in working Tris HCl buffer for 2 minutes after each treatment.
- 10 Primary antibody was applied to the sections; N-cadherin (Clone 5D5, Abcam Cambridge, United Kingdom, ab98952, 1/200 dilution for 60 minutes) and Type-IV collagen (Clone CIV22 Dako, Denmark, Catalogue no. M0785, 1:100 for 90 minutes). A negative control was also used in each run with isotyped matched immunoglobulin IgG1 (clone X0931 Dako Denmark A/S). Lung sections from previous biobank were used as positive controls for confirmation of staining.
- 11 Sections were rinsed well using Tris HCl (thrice, 5 mins each).
- 12 Applied EnVision+ system-HRP labelled polymer (catalogue number K4001; Dako, Denmark A/S) reagent to sections for 30 mins and rinsed with Tris HCl buffer.
- 13 DAB+ (catalogue number K3468; Dako, Denmark A/S) was applied to sections for 10 minutes and rinsed well using Tris HCl (twice) followed by distilled water.
- 14 The slides were taken out of Autostainer and put in Mayers hematoxylin to elaborate nuclei for 3-5 minutes, then rinsed in running water.
- 15 Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds, and rinsed well in running water.
- 16 Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each).
- 17 Clearing was done in two changes of xylene (2 minutes each).
- 18 Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.5.5 General procedure for TGFβ1, pSmad2/3 and pSmad7 staining

- 1 Paraffin blocks were sectioned at 3µms after cooling in -20 freezer or on ice blocks 5-10 minutes.

- 2 Two sections (approximately 3µm thick) separated by 40-50 micron (approximately 10 sections) were cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
- 3 Slides were labelled using printed labels and marked on the edges with wax pen.
- 4 Dewaxing of sections was done in “xylene” (twice for 5 minutes each) in fume hood.
- 5 Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections well in running tap water (2 minutes).
- 6 Antigen retrieval was performed in PT Link (Dako, Denmark A/S): for TGFβ1 (no heat retrieval): for pSmad2/3, sections were kept at 97°C at low pH buffer for 30 minutes: for pSmad7, a temperature of 97°C at high pH buffer for 30 minutes was used.
- 7 After antigen retrieval, the slides were cooled down to room temperature and transferred to Dako Autostainer Plus (Dako, Denmark, A/S), in which a program template for staining was made.
- 8 For TGFβ1, pSmad2/3 and pSmad7, sections were placed in 3% H₂O₂ in distilled water for 15 minutes and washed in distilled water for 2 minutes to block endogenous peroxidase activity.
- 9 Sections were rinsed in working Tris HCl buffer for 2 minutes after each treatment.
- 10 Primary antibody was applied to the sections; TGFβ1 (Clone TB21, Abcam Cambridge, United Kingdom, ab27969, 1/6000 dilution after blocking with Dako serum block (X0909), pSmad2/3 (Santa-Cruze, California, USA, SC-11769R, 1/100 dilution for 60 minutes) and pSmad7 (Santa-Cruze, California, USA, SC-101152, 1/100 dilution for 60 minutes). A negative control was also used in each run with isotyped matched immunoglobulin IgG1 (clone X0931 Dako Denmark A/S). Lung sections from previous biobank were used as positive controls for confirmation of staining.
- 11 Sections were rinsed well using Tris HCl (thrice, 5 mins each).

- 12 Applied EnVision+ system-HRP labelled polymer (catalogue number K4001; Dako, Denmark A/S) reagent to sections for 30 mins and rinsed with Tris HCl buffer.
- 13 DAB+ (catalogue number K3468; Dako, Denmark A/S) was applied to sections for 10 minutes and rinsed well using Tris HCl (twice) followed by distilled water.
- 14 The slides were taken out of Autostainer and put in Mayers haematoxylin to elaborate nuclei for 3-5 minutes, then rinsed in running water.
- 15 Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds, and rinsed well in running water.
- 16 Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each).
- 17 Clearing was done in two changes of xylene (2 minutes each).
- 18 Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried on hotplate overnight.

3.5.6 General procedure for β -catenin, Snail1 and Twist staining

- 1 Paraffin blocks were sectioned at 3 μ m after cooling in -20 freezer or on ice blocks 5-10 minutes.
- 2 Two sections (approximately 3 μ m thick) separated by 40-50 micron (approximately 10 sections) were cut and put on each slide [3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50°C)] or Dako FLEX IHC microscopic slides (Code K8020, Dako Denmark A/S). Slides were dried on hotplate at 56°C for one hour and overnight at 37°C.
- 3 Slides were labelled using printed labels and marked on the edges with wax pen.
- 4 Dewaxing of sections was done in "xylene" (twice for 5 minutes each) in fume hood.
- 5 Hydration to water was carried out using 100% ethanol followed by 95% then 70% (3 minutes each) followed by rinsing the sections well in running tap water (2 minutes).

- 6 Antigen retrieval was performed in PT Link (Dako, Denmark A/S): for β -catenin, sections were kept at 97°C at high pH buffer for 15 minutes: for Snail1 sections were kept at 97°C at high pH buffer for 15 minutes: for Twist, a temperature of 97°C at low pH buffer for 15 minutes was used.
- 7 After antigen retrieval, the slides were cooled down to room temperature and transferred to Dako Autostainer Plus (Dako, Denmark, A/S), in which a program template for staining was made.
- 8 For β -catenin, Snail1 and Twist, sections were placed in 3% H₂O₂ in distilled water for 20 minutes and washed in distilled water for 2 minutes to block endogenous peroxidase activity.
- 9 Sections were rinsed in working Tris HCl buffer for 2 minutes after each treatment.
- 10 Primary antibody was applied to the sections; anti- β -catenin (Clone 12F7 Ab 22656, Abcam Cambridge, United Kingdom, 1/500 dilution for 90 minutes), Snail1 (Abcam Cambridge, United Kingdom, ab180714, 1/200 dilution for 90 minutes after blocking with Dako serum block (X0909) and Twist (Ab50581 Abcam Cambridge, United Kingdom, 1/500 dilution for 90 minutes). A negative control was also used in each run with isotyped matched immunoglobulin IgG1 (clone X0931 Dako Denmark A/S). Lung sections from previous biobank were used as positive controls for confirmation of staining.
- 11 Sections were rinsed well using Tris HCl (thrice, 5 mins each).
- 12 Applied EnVision+ system-HRP labelled polymer (catalogue number K4001; Dako, Denmark A/S) reagent to sections for 30 mins and rinsed with Tris HCl buffer.
- 13 DAB+ (catalogue number K3468; Dako, Denmark A/S) was applied to sections for 10 minutes and rinsed well using Tris HCl (twice) followed by distilled water.
- 14 The slides were taken out of Autostainer and put in Mayers hematoxylin to elaborate nuclei for 3-5 minutes, then rinsed in running water.
- 15 Sections were placed in approximately 400 ml of water with 6-8 drops of ammonia for 30 seconds, and rinsed well in running water.
- 16 Dehydration was carried out in 95% ethanol, then two changes of 100% ethanol (2 minutes each).
- 17 Clearing was done in two changes of xylene (2 minutes each).

18 Sections were mounted in Depex using Dako Coverslipper (Dako, Denmark A/S) and dried overnight, on hotplate.

3.6 Sections quantification

Computer-assisted image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot Insight-12 (Spot Imaging Solutions, USA) digital camera and Image Pro Plus 7.0 software (Media Cybernetics, USA). As many non-overlapping pictures as possible were taken of the tissue from the areas of interest: (for studies involving endobronchial biopsies it was mainly epithelium, Rbm and lamina propria; for larger airways, and epithelium, Rbm and whole airway wall thickness for small airways <2mm diameter. For Chapter 7 involving tumor analysis, central and peripheral leading edge areas of NSCLC. Image randomization was done by using statreck.com with their random number generator. Randomly selected images (five for EBBs and eight for resected tissue) were then taken from the total number of images and used for the desired measurements.

3.6.1 Quantification of EGFR, S100A4, Vimentin and Type-IV collagen expression

EMT bio-markers in airways: Epithelial EGFR expression in large and small airways was expressed as the percentage of epithelium with EGFR expression. Basal epithelial S100A4 and vimentin expression in large and small airways was quantified and normalized by length of airway reticular basement membrane (Rbm). Similarly, S100A4 and vimentin expressing cells in airway Rbm were quantified and normalized by length of Rbm. I quantified the degree of Rbm fragmentation by measuring the length of linear clefts, and expressed these as percentage of total Rbm length. I also quantified number of Type-IV expressing blood vessels in Rbm in large and small airways and again normalized by length of Rbm (Chapter 4).

EMT biomarkers in NSCLC: Evaluation of cell staining for EGFR, S100A4, vimentin and N-cadherin was performed both in the central and peripheral leading edge of NSCLC, in accordance with the “immunoreactive score” (IRS) with modification: $IRS = SI \text{ (staining intensity)} \times PP \text{ (percentage of positive cells)}$. In the modified IRS scoring

SI was determined as 0, negative; 1, weak; 2, moderate; and 3, strong and PP was ranged from 0-100% in order to get a wider range for analysis. TNM staging was done on the basis of their final recorded histopathological evaluation. TNM stage was scored according to TNM factor (T x N x M); T (Tumor size), N (Lymph node involvement) and M (Metastasis - Pleural/lympho-vascular/haematogenous). The majority of patients were in stage T1 or T2 plus N1. Even so, because of some patients were at T3 or N2 or M1 with pleural or lymphatic involvement, I was able to have quite a wide range of scoring for correlations (Chapter 7).

3.6.2 Quantification of TGFβ1, pSmad2/3 and pSmad7 expression

Basal epithelial TGFβ1, pSmad2/3 and pSmad7 expression was quantified and normalized over length of the airway Rbm. Similarly, TGFβ1, pSmad2/3 and pSmad7 expressing cells and vessels in airway Rbm were quantified and normalized over length of Rbm. Using the image analyser, I also quantified pSmad2/3 and pSmad7 expressing cells and vessels up to 150μm deep into the lamina propria and results presented as cells per mm² of lamina propria (Chapter 5).

3.6.3 Quantification of β-catenin, Snail1 and Twist expression

Again, basal epithelial β-catenin, Snail1 and Twist expression was quantified and normalized over length of the airway Rbm. Similarly, β-catenin, Snail1 and Twist expressing cells in airway Rbm were quantified and normalized over the length of Rbm. Using the image analyser, I also quantified β-catenin, Snail1 and Twist expressing cells up to 150μm deep into the lamina propria and results presented as cells per mm² of lamina propria (Chapter 6).

For all such work done throughout this thesis, all slides were coded and randomized by an independent person (Mr. Steven Weston, our very experienced laboratory manager) and then counted in a single batch by myself fully blinded to subject and diagnosis. Quality assurance on randomly selected slides was provided by a professional academic pathologist (Professor H.K. Muller).

3.7 Statistical analysis

In chapter 4, there were two separate comparisons involved namely, large versus small airways; and small airway versus control small airways Normally distributed data (Shapiro-Wilk test) for EMT bio-markers in the airways (Chapter 4) and NSCLC

(chapter 8) were expressed as means with 95% confidence intervals (CI). Straight comparisons were undertaken using paired *t* tests using Graph Pad Prism (Graph Pad Software Inc. United States of America).

Cross-sectional multi-group data on TGF β 1, pSmad2/3, pSmad7 (Chapter 5), β -catenin, Snail1 and Twist, (Chapters 6) were non-normally distributed, and so data were presented as medians and ranges; Non- parametric analyses of variance (ANOVA) were performed using the Kruskal-Wallis Test to detect any overall difference among study groups, followed by Dunn's multiple comparison test to specify which groups were different.

I also performed regression analysis for nearly all biomarker expressions level against FEV₁, FERatio, FEF25-75% (for small airway assessment) in the COPD groups, using Spearman's rank test. Linear regression analyses for potential confounders were undertaken and differences between groups in gender and age were found to be non-contributory throughout.

All statistical analyses were performed using SPSS (statistics version 20.0, IBM Corporation, Armonk, NY, USA) for Windows 10.0 and Graph Pad Prism 6.0 (Graph Pad Software Inc. United States of America) and a $p < 0.05$ was considered statistically significant.

Chapter 4

4. Epithelial mesenchymal transition (EMT) in smokers: large versus small airways; and relation to airflow obstruction

4.1 Introduction

As reviewed in detail in chapter 2, recent observations have emphasized Rbm fragmentation, cellularity and hyper-vascularity in large airway biopsies from smokers and COPD patients (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010; Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2011; Soltani, Muller, et al. 2012) as evidence of EMT Type 3. There is limited evidence that EMT is also active in small airways (Milara, Peiro, et al. 2013; Sohal, S. S. & Walters 2013). Angiogenesis in the Rbm is specifically smoking related (Hiroshima, K. et al. 2002; Walters, E. H. et al. 2008) while other features are most marked in COPD (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010). To my knowledge, no reported studies in human resected lung tissue exploring individual type of EMT have been done, nor observations on the relationship between EMT activity in small airways and airflow obstruction in COPD patients.

Thus our previous published work used large airway endoscopic biopsies (Soltani, Muller, et al. 2012). This encouraged me to design the present study in order to compare the degree of EMT activity in small versus matching large airways and also the type of EMT in each compartment, using lung tissue resected from smokers undergoing lung resection for lung cancer. I have also, for the first time, explored relationships between epithelial activation and EMT activity with the degree of airflow obstruction in this group selected to have a wide variation in chronic airflow limitation (CAL).

4.2 Overview of materials and methods

The demographics of subjects involved are detailed in Table 4.1. I employed classical immune-histochemical methods, described in chapter 3 to evaluate an epithelial activation marker (EGFR), EMT biomarkers (S100A4 and vimentin), and vascularity marker (Type-IV collagen) in the large and small airways in resected tissue.

| Groups (numbers) | CAL (n=18) | Normal (n=9) |
|------------------------------|-----------------|---------------|
| Male/Female | 13/5 | 6/3 |
| Age (years) | 64(58.7-69.2) | 52(42.4-63.3) |
| Smoking pack years (Range) | 39(24.1-40.1) | N/A |
| FEV ₁ % predicted | 82 (76-89.4) | N/A |
| FEV/FVC % (Range) | 69(66.9-73.1) | N/A |
| FEF25-75% predicted | 48.5(39.9-57.1) | N/A |

Table 4.1: Demographic details and Lung Function data of CAL Patients and Control Subjects.

Data expressed as mean and CI95%;

CAL=Chronic Airflow Limitation

FEV₁= Forced Expiratory Volume in 1 second

FVC =Force Vital Capacity

FEF = Force Expiratory Flow

4.3 Results

The overall results of different groups are presented in Tables 4.2 and 4.3.

4.3.1 EGFR expression in Epithelium

A greater proportion of the airway epithelium stained for EGFR in large airways of CAL patients (mean 11.4%, CI 9.9-12.8) compared to CAL small airway (mean 6.8%, CI 5.5-8.1) ($p<0.001$) (Figure 4.1). There was also a marked difference between small airways in the CAL group and the normal small airways (mean 0.6%, CI 0.3-0.8) ($p<0.001$) (Figure 4.2).

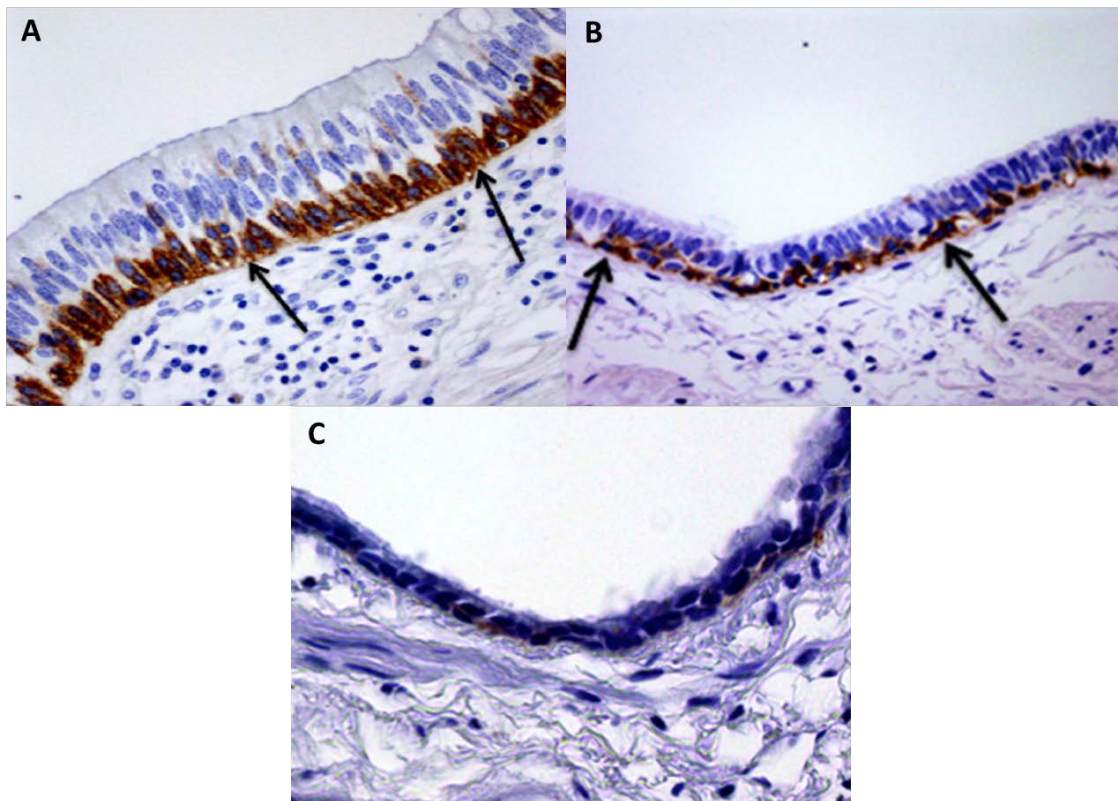


Figure 4-1: EGFR Expression

A) Large airway and B) Small airway tissue from patient with CAL and C) Normal small airways. Black arrows indicate airway epithelium (chronic airflow limitation) stained positive for EGFR (epithelial activation marker). Original magnification, $\times 400$. Scale bar = 50 μm .

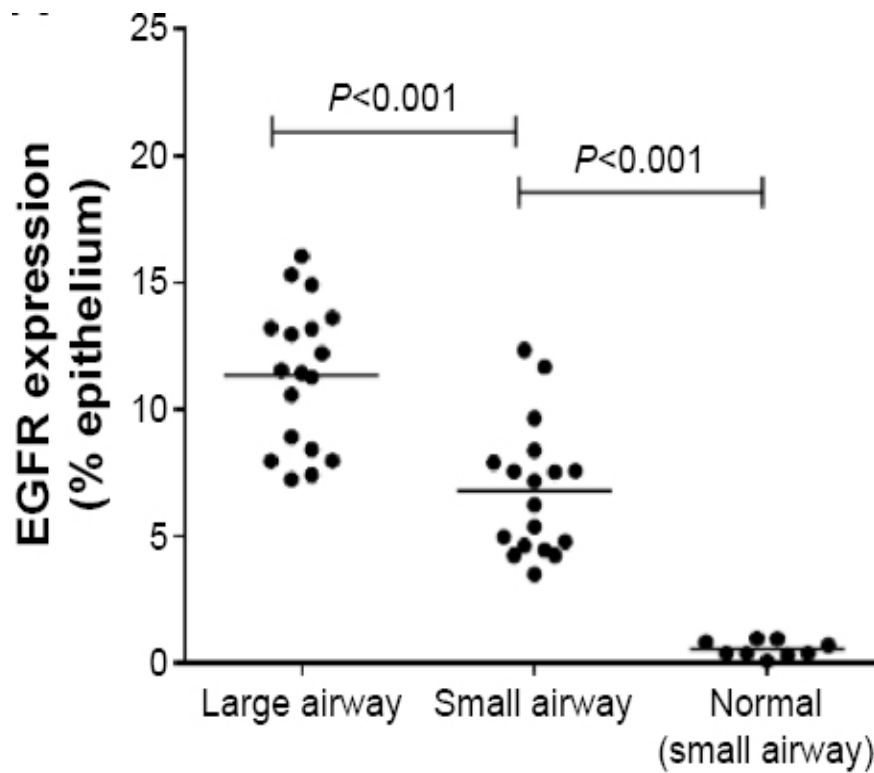


Figure 4-2: Percentage of airway Epithelium showing EGFR Expression

in CAL patients stained for EGFR compared with CAL small airway and normal control small airway.

4.3.2 Fragmentation in the reticular basement membrane (Rbm)

Fragmentation of the Rbm was more marked in large airways from CAL subjects (mean 27.9%, CI 24.3-31.6) than in their small airways (mean 14.9%, CI 13-16.9) ($p<0.001$) (Figure 4.3). Small airways from CAL subjects showed significantly greater fragmentation than in normal small airways (mean 2.4%, CI 2.1-2.7) ($p<0.001$) (Figure 4.4)

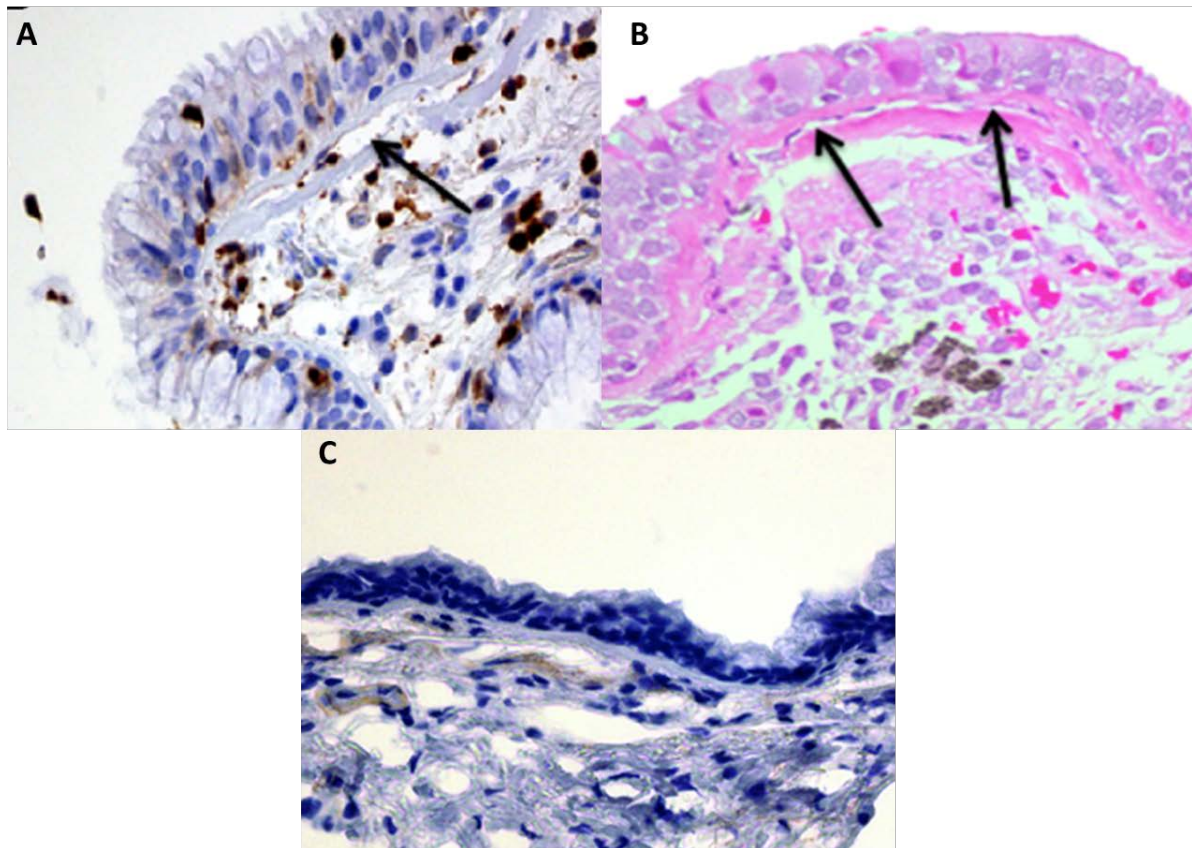


Figure 4-3: Rbm Fragmentation

A) Large airway and B) small airway tissue from a patient with CAL and C) Normal small airways. Black arrows indicate Rbm fragmentation. Original magnification, $\times 400$. Scale bar = 50 μm .

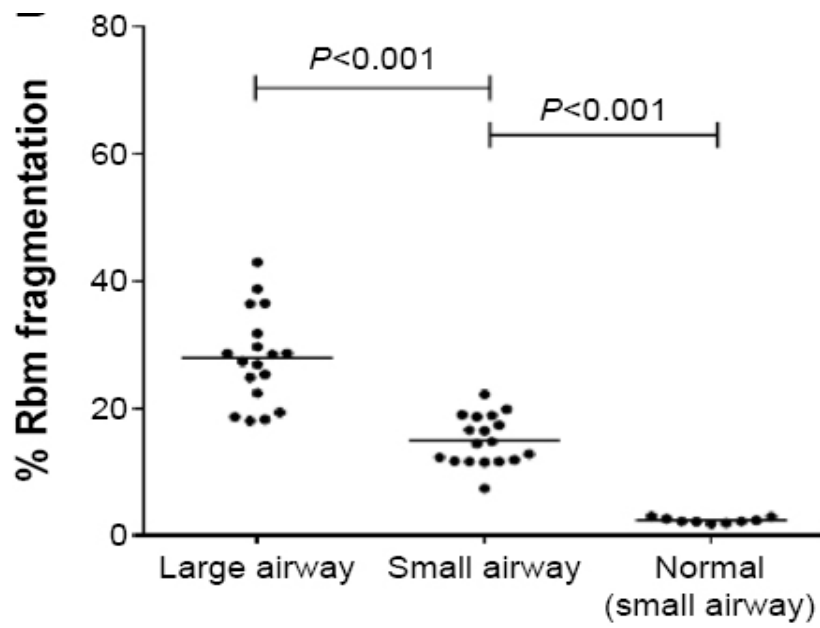


Figure 4-4: Percentage of Rbm fragmentation.

(Total length of clefts as percentage of the total length of Rbm) in large airway in CAL patients versus CAL small airway and in normal control small airway.

4.3.3 S100A4 and vimentin positive cells in the basal epithelium

I observed S100A4 and vimentin staining in the basal layer of the epithelium in both large and small airways in CAL, but with considerably more marked expression in large airways ($p < 0.001$ for each) (Figure 4.5) (S100A4, mean 42.9 cells/mm³, CI 38.5-47.4; vimentin, mean 69.7 cells/mm³, CI 60.1-79.3) than in small airways (S100A4, mean 25.9 cells/mm³, CI 22.8-28.9; vimentin, mean 41.5 cells/mm³, CI 35.8-47.4) (Figure 4.6 and 4.7). In turn there was significantly greater expression in CAL small airways than in normal control small airways (S100A4, mean 6.4 cells/mm³, CI 4.7-7.9; vimentin, mean 8.2 cells/mm³, CI 6.9-9.4) (for both $p < 0.001$) (Figure 4.7).

4.3.4 S100A4 and vimentin positive cells in the Rbm

In comparison to CAL large airways (S100A4, mean 24.1 cell/mm³, CI 22.2-25.9; vimentin, mean 36.1 cells/mm³, CI 32.8-39.5) (Figure 4.5) there was slightly but significant less expression of these mesenchymal markers in small airways (S100A4, mean 13.9 cells/mm³, CI 12.5-15.3; vimentin, mean 27.8 cells/mm³, CI 25.4-30.2) (for both $p < 0.001$) (Figure 4.6 and 4.7). However, once again expression was greater in the small airways in CAL compared to normal control small airways (S100A4, mean 5.3 cells/mm³, CI 4.1-6.5; vimentin, mean 8.1 cells/mm³, CI 6.7-9.5) (for both $p < 0.001$) (Figure 4.7).

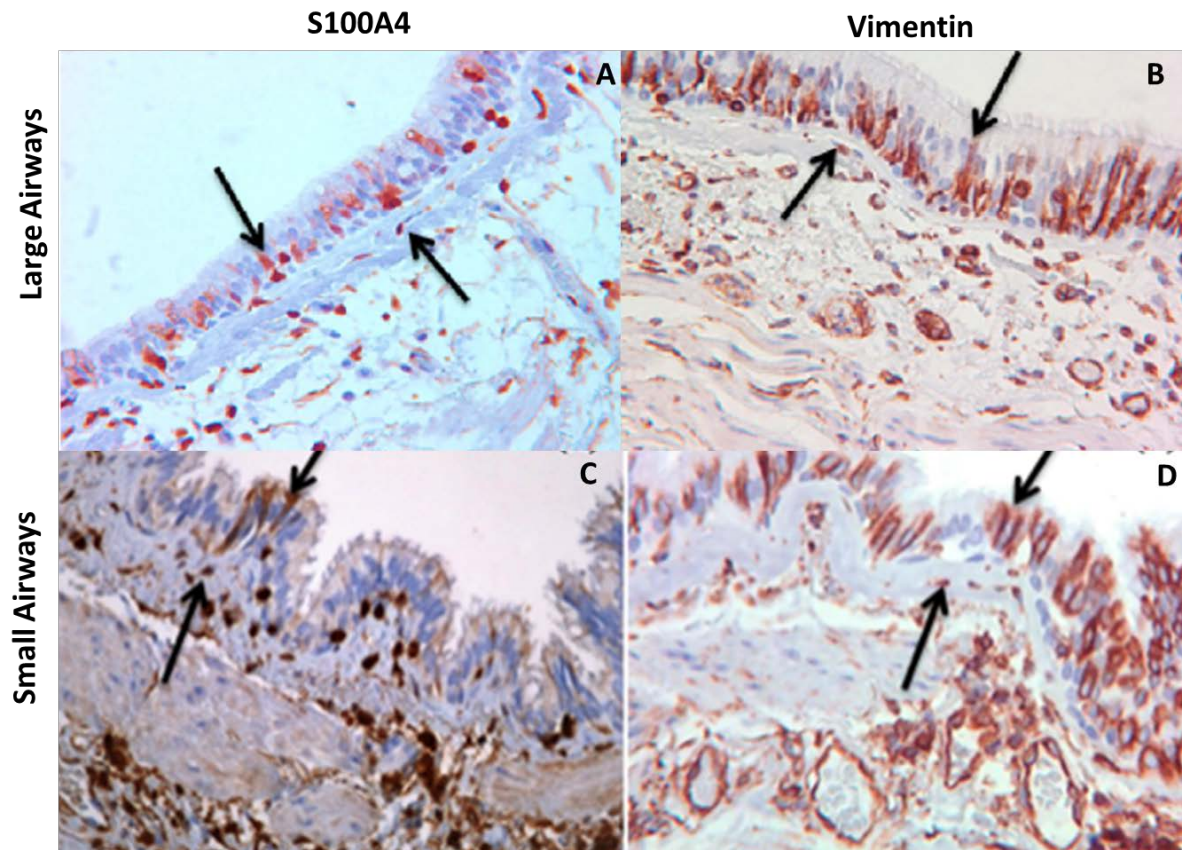


Figure 4-5: S100A4 and Vimentin Expression

A&B) large airways and C&D) Small airways. Black arrows indicate: (A&C) S100A4 and (B&D) vimentin positive cells in epithelium and fragmented Rbm. Original magnification, $\times 400$. Scale bar = $50\ \mu\text{m}$.

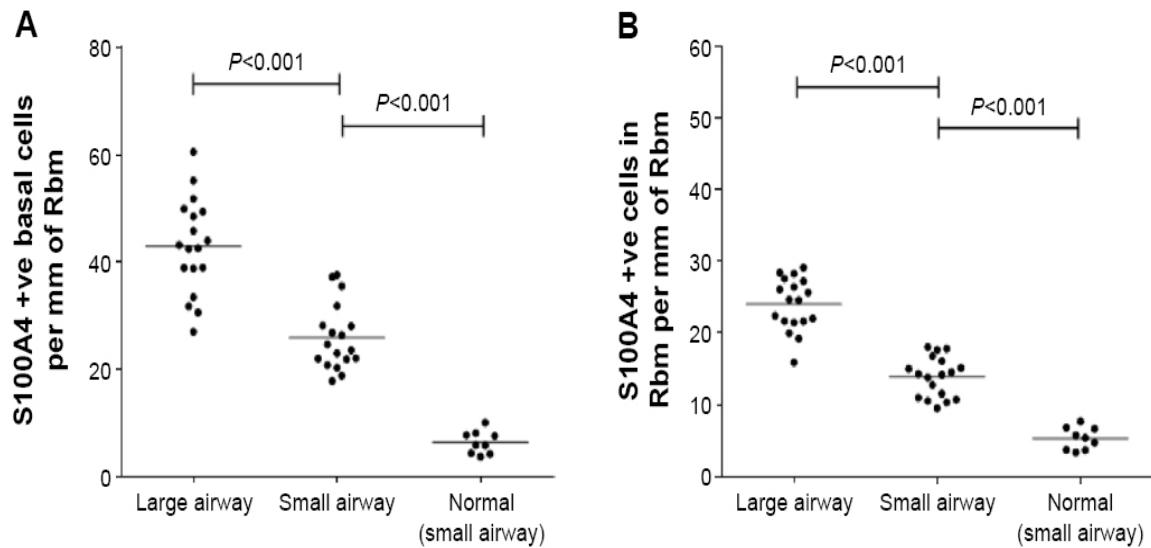


Figure 4-6: Comparison of S100A4 Expression

In the basal epithelial cells of large airway in CAL patients, with small airway of CAL patients and normal control small airway. (B) Comparison of S100A4 expression in Rbm of large airway in CAL patients, with small airway of CAL patients and normal control small airway.

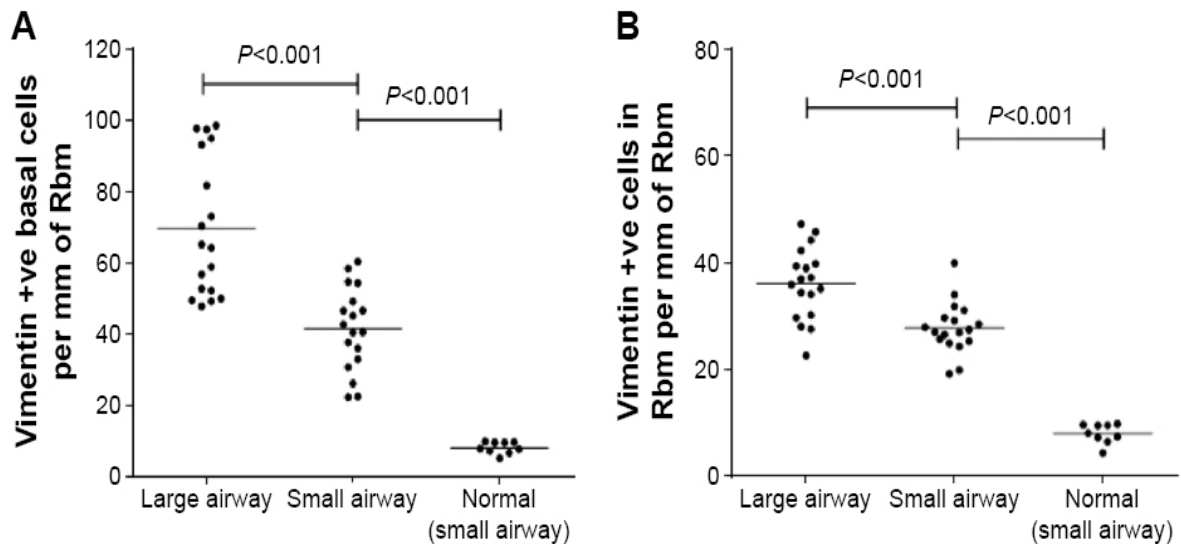


Figure 4-7: Comparison of vimentin Expression

In basal epithelial cells in CAL large airway versus CAL small airways and in normal control small airway. (B) Comparison of number of vimentin-positive cells in Rbm in CAL large airway versus CAL small airway and normal control small airway.

4.3.5 Blood vessels in the Rbm

Hyper vascularity of the Rbm was found only in CAL large airways (mean 8.5 vessels per mm of Rbm, CI 7.6-9.4) (Figure 4.8), and not in the small airways either in CAL (mean 0.2 vessels per mm of Rbm, CI 0.05-0.3) ($p<0.001$) or the control group (mean 0.05 vessels per mm of Rbm, CI 0.02-0.13) ($p=NS$) (Figure 4.9).

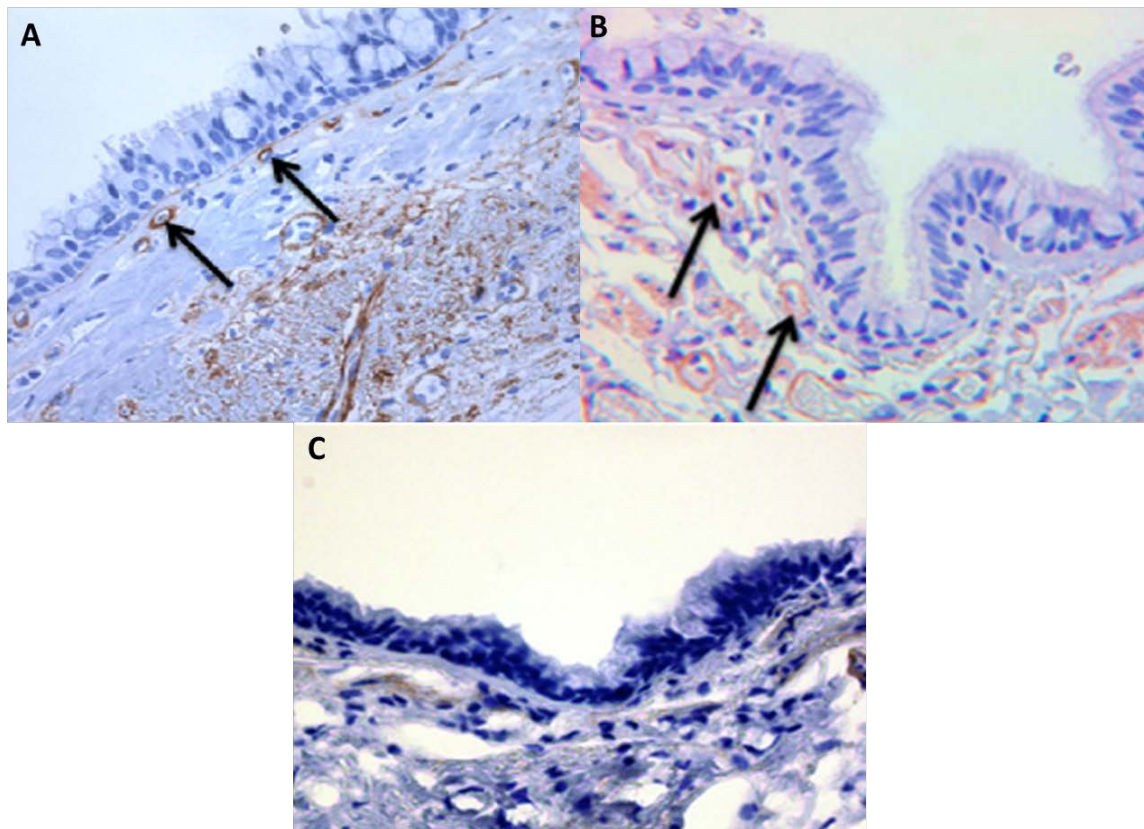


Figure 4-8: Type-IV expression by blood vessels

(A) Black arrows indicate hyper vascularity of the Rbm with Type-IV collagen-stained blood vessels in the Rbm (EMT Type 3). (B) In small airway, vessels are seen only in lamina propria (black arrows) while Rbm is devoid of vessels. C) Normal small airways. Original magnification, $\times 360$. Scale bar = 50 μm .

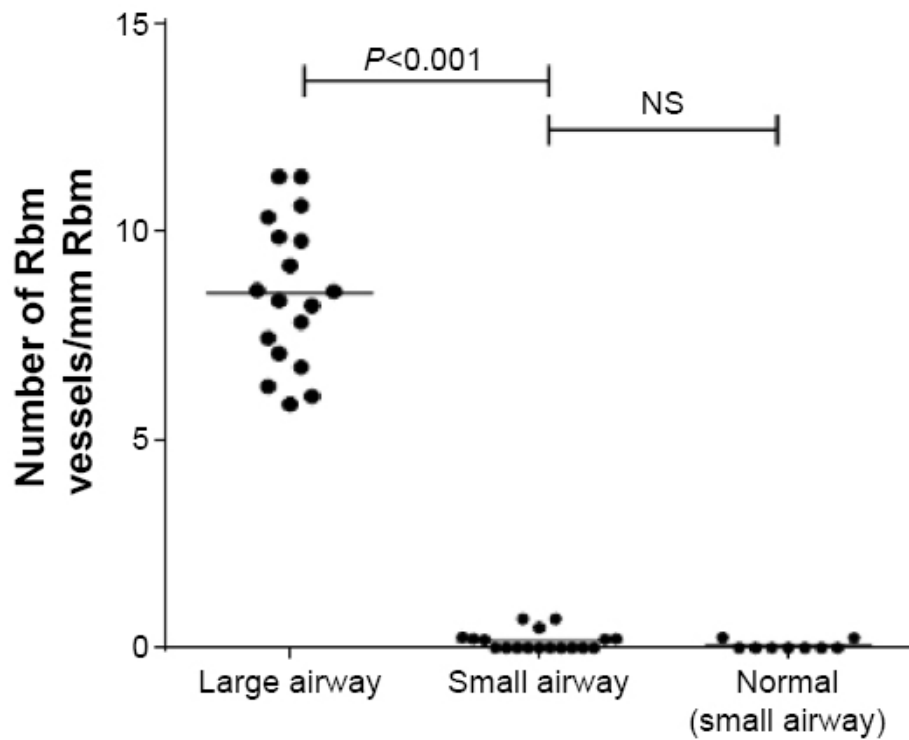


Figure 4-9: Comparison of number of blood vessels in large airway (RBM) of CAL compared with CAL small airway and normal control small airway.

| Indices | AIRWAY (CAL) | | Mean Difference (95% CI) | Small airway (Normal Control) |
|--|---------------------|---------------------|---------------------------------------|----------------------------------|
| | Large | Small | | |
| EGFR (Epithelium) (%) | 11.4 (9.9-12.8) | 6.8 (5.5-8.1) | 4.6(2.6 to 6.5) <i>P</i> <0.001 | 0.6 (0.3-0.8) <i>P</i> <0.001 |
| S100A4 (Epithelium) (per mm of rbm) | 42.9 (38.5-47.4) | 25.9 (22.8-28.9) | 17(11.4 to 22.6) <i>P</i> <0.001 | 6.4 (4.7-7.9) <i>P</i> <0.001 |
| S100A4 (Rbm) (per mm of rbm) | 24.1 (22.2-25.9) | 13.9 (12.5-15.3) | 10.2(7.7 to 12.6) <i>P</i> <0.001 | 5.3 (4.1-6.5) <i>P</i> <0.001 |
| Vimentin (Epithelium) (per mm of rbm) | 69.7 (60.1-79.3) | 41.5 (35.8-47.4) | 28.2(16.5 to 39.8) <i>P</i> <0.001 | 8.2 (6.9-9.4) <i>P</i> <0.001 |
| Vimentin (Rbm) (per mm of rbm) | 36.1 (32.8-39.5) | 27.8 (25.4-30.2) | 8.3(4.1 to 12.6) <i>P</i> <0.001 | 8.1 (6.7-9.5) <i>P</i> <0.001 |
| Fragmentation (Rbm) (per mm of rbm) | 27.9 (24.3-31.6) | 14.9 (13-16.9) | 13(8.72 to 17.26) <i>P</i> <0.001 | 2.4 (2.1-2.7) <i>P</i> <0.001 |
| Vessels (Rbm) (per mm of rbm) | 8.5 (7.6-9.4) | 0.2 (0.05-0.3) | 8.3(7.4 to 9.1) <i>P</i> <0.001 | 0.05 (0.02-0.13) <i>P</i> =NS |

Table 4.2: Comparison of different pathological indices in CAL Large and Small airways

(Data expressed as mean and CI95%).

| Indices | Small Airways | | Mean Difference (95% CI) |
|--|---------------------|---------------------|--|
| | (CAL) | (Normal Control) | |
| EGFR (Epithelium) | 6.8 (5.5-8.1) | 0.6 (0.3-0.8) | 6.2 (3.8 to 8.6) <i>P</i> <0.001 |
| S100A4 (Epithelium) (per mm of rbm) | 25.9 (22.8-28.9) | 6.4 (4.7-7.9) | 19.5 (12.7 to 26.5) <i>P</i> <0.001 |
| S100A4 (Rbm) (per mm of rbm) | 13.9 (12.5-15.3) | 5.3 (4.1-6.5) | 8.6 (5.6 to 11.6) <i>P</i> <0.001 |
| Vimentin (Epithelium) (per mm of rbm) | 41.5 (35.8-47.4) | 8.2 (6.9-9.4) | 33.3 (19.1 to 47.6) <i>P</i> <0.001 |
| Vimentin (Rbm) (per mm of rbm) | 27.8 (25.4-30.2) | 8.1 (6.7-9.5) | 19.7 (14.5 to 24.9) <i>P</i> <0.001 |
| Fragmentation (Rbm) (per mm of rbm) | 14.9 (13-16.9) | 2.4 (2.1-2.7) | 12.5 (7.3 to 17.8) <i>P</i> <0.001 |
| Vessels (Rbm) (per mm of rbm) | 0.2 (0.05-0.3) | 0.05 (0.02-0.13) | 0.1 (-1 to 1.2) <i>P</i> = NS |

Table 4.3: Comparison of different pathological indices in CAL small airway and normal control small airway.

(Data expressed as mean and CI95%).

4.3.6 Regression Data

In CAL subjects there were significant relationships between increasing airflow obstruction and increasing epithelial activation (EGFR expression) (Figure 4.10) and enhanced S100A4 expression in both epithelial basal cells and in Rbm cells (Figure 4.11), generally most marked for small airways pathology and for small airway obstruction (FEF₂₅₋₇₅ % predicted). There was quite a dichotomy between these relationships and the absence of such relationships for Rbm fragmentation and vimentin expression (Table 4.4).

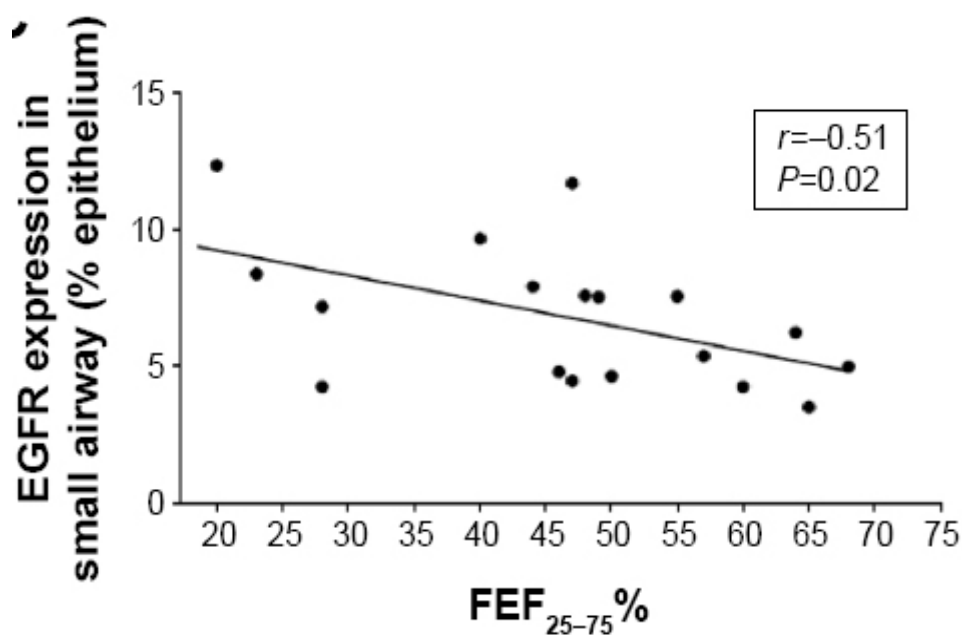


Figure 4-10: Correlation between EGFR (% epithelium) in small airway and FEF₂₅₋₇₅%.

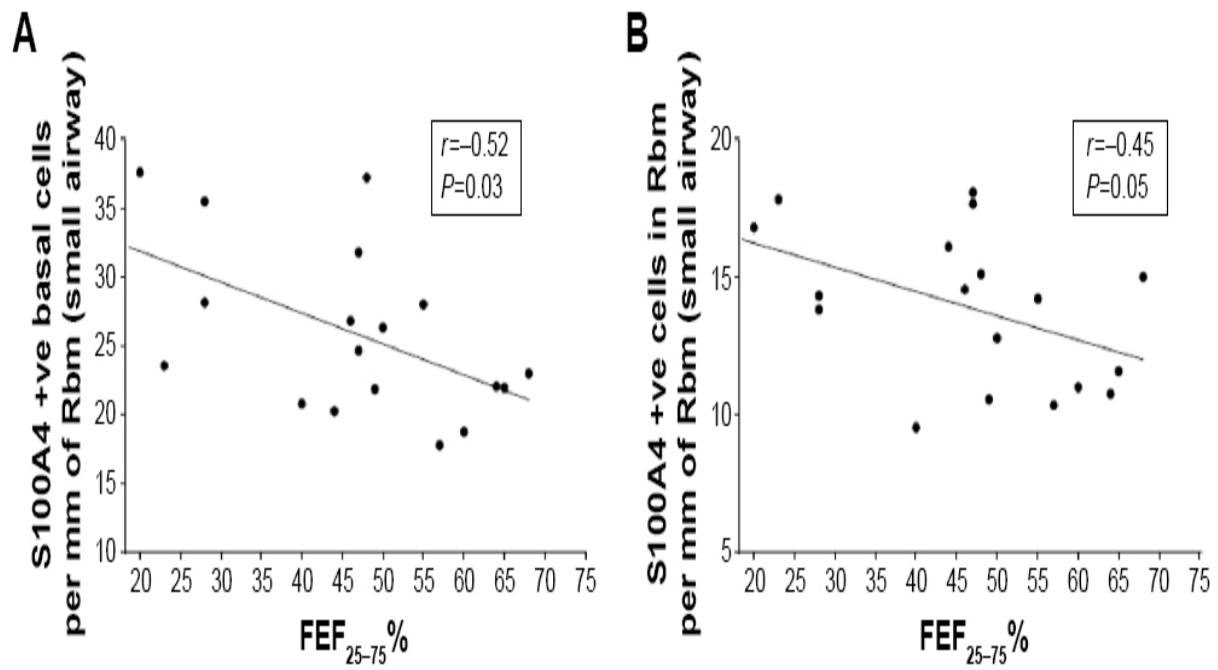


Figure 4-11: Correlation between small airways S100A4 expression and FEF25–75%

A) Basal epithelial cells B) Rbm

| Indices | Large Airways pathology | | | | Small Airways pathology | | | |
|----------------------------|-------------------------|-------------|-----------------------|-------------|-------------------------|-------------|-----------------------|-------------|
| | FEV ₁ / FVC | | FEF _{25-75%} | | FEV ₁ / FVC | | FEF _{25-75%} | |
| | r | p | r | p | r | p | r | p |
| EGFR | -0.11 | 0.65 | 0.01 | 0.96 | -0.46 | 0.06 | 0.51 | 0.02 |
| S100A4 – Epi | -0.44 | 0.06 | -0.44 | 0.06 | -0.44 | 0.06 | -0.52 | 0.03 |
| S100A4 - Rbm | -0.46 | 0.05 | -0.53 | 0.02 | -0.60 | 0.01 | -0.45 | 0.05 |
| Vimentin - Epi | 0.08 | 0.75 | 0.17 | 0.51 | 0.08 | 0.73 | -0.21 | 0.40 |
| Vimentin - Rbm | 0.03 | 0.90 | -0.36 | 0.14 | -0.16 | 0.54 | -0.14 | 0.59 |
| Fragmentation (Rbm) | -0.15 | 0.53 | -0.09 | 0.73 | -0.11 | 0.66 | -0.02 | 0.95 |
| Vessels (Rbm) | -0.13 | 0.58 | 0.02 | 0.92 | 0.09 | 0.71 | -0.08 | 0.75 |

Table 4.4: Linear regression of pathological data from CAL subjects versus spirometric indices.

4.4 Discussion

This study sought to analyse the expression of epithelial activation and classic EMT structural and mesenchymal biomarkers and also vascular changes, in matching large and small airways from smoking patients with airflow obstruction (CAL). I have extended previously demonstrated findings where compared to normal controls, smokers highly expressed these bio-markers, but this was greatest in COPD (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010). Here I show that small airways from a group of CAL patients also demonstrated increased EGFR expression as well as active EMT significantly above normal, but uniformly less so than in large airway. However, in small airways, EMT changes were not associated with hyper-vascularity i.e. could be considered as the pro-fibrotic Type-2 EMT rather than the more malignancy-associated Type-3 EMT.

In both large and small airways in this CAL group, staining for S-100A4 and vimentin was focused in the basal cell layer of the epithelium. This may highlight the likelihood of these cells being especially involved in undergoing transition to a mesenchymal phenotype. Similar positive staining of cells in the clefts of the disrupted Rbm further strengthens this concept of transition and migration of these basal cells. We have previously shown that the Rbm cells are not immune or inflammatory cells, and that they do strongly express matrix metalloprotein-9 (MMP-9), i.e. with proteolytic capacity to digest their way through the lamina propria, and so likely causing the Rbm fragmentation (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010; Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2011).

The differentiation into Type-2 EMT in small airways (<2mm internal diameter) is consistent with fibrosis and obliteration here. Classically more cancer develops in large airways (Hogg et al. 2004; Prudkin et al. 2009; Yanai et al. 1992), especially for the squamous cell variety where Type-3 EMT predominates. Myofibroblast accumulation accompanies the damage to small airways in COPD (Burgel et al. 2011). The origin of these myofibroblasts is not fully understood, but our data suggest that they might be derived from EMT. Endothelial mesenchymal transition (EndoMT), pericytes transformation or stroma cells are other possible sources (Milara, Serrano, et al. 2013; Scotton & Chambers 2007). Further work is needed to delineate these possibilities.

I have now shown that EMT is active in small airways and that at least S100A4 expression is associated with airflow obstruction suggesting that this fibroblast marker may indeed be relevant to airway fibrosis. Interestingly, EMT is also thought to be involved in development of idiopathic pulmonary fibrosis (Coward, Deacon & Pang 2013), and since this is also frequently smoking related, it suggests a certain pathological unity with the process in the small airways. That's in detail fibroblast subtypes and related changes in extracellular matrix (ECM) would be my future goal as well.

In agreement with our findings, Milara and colleagues also observed up regulation of EMT biomarkers in small airways in COPD (Milara, Peiro, et al. 2013). Such EMT changes could also be induced in culture by cigarette smoke. Our study takes this further by quantifying Rbm structural changes, comparing large and small airway and characterising Type-2 versus Type-3 EMT. Milara et al also implicated increasing cyclic AMP in EMT activity in the airways (Milara et al. 2014), while others implicated an urokinase plasminogen activator receptor dependant signalling pathway (Wang, Q et al. 2013) or a muscarinic mechanism (Yang, K et al. 2014).

The main strength of this study is that I directly investigated human tissue from CAL-affected individuals. Further, I have a sufficiently robust number of participants with good spread of airflow obstruction in large and small airways. Some limitations are however inevitable, especially availability of normal control small airway samples and international collaboration has been needed to provide this. I have focused on empiric but classically described (Pain, M. et al. 2014) "down-stream" phenotypic manifestation of EMT i.e. structural changes and protein expression. I will follow up with detailed comparative analysis of more upstream pathways and expression of EMT-related transcription factors in large versus small airway whole tissue.

Finally, due to the number of patients available to analyse, I have treated those with frank COPD (FER less than 70%) and those only with abnormal small airway function (scalloping of the expiratory limb of the volume loop and low FEF_{25-75%}) as a single group. The advantage that this gave to me is that I had a wide range of physiological values for regression analyses, which have produced extremely interesting results. Our current group of cancer patients contained only four subjects without evidence of any airflow obstruction. They were not included in this chapter as there were not

sufficient numbers for a non-CAL group. They were included in Chapter 7 (using all the cancer subjects). It is interesting how relatively uncommon completely physiologically normal smoking cancer patients seems to be, but it is also noteworthy that they do seem to have EMT changes in both large and small airways, though less marked. Indeed, had their data been added to our regressions, these would have been even more significance because of a broader range of data provided.

In conclusion, my data provide evidence for active EMT in small as well as the previously demonstrated large airways of smokers with CAL. On the basis of Rbm hyper-vascularity, the EMT in large airways is characteristic of so-called Type-3 EMT, while Type-2 EMT is now shown to be predominant in small airways. This is consistent with the major pathologies in the two compartments: in large airways the process might especially form a microenvironment for the development of lung cancer, while Type-2 EMT is thought to be especially pro-fibrotic, and might be more related to small airway fibrosis, obstruction and obliteration. The relationship seen between the fibroblastic marker S100A4 and airflow obstruction would definitely open up a very significant potential to explain the exact pathogenesis of COPD and its role in fibrosis, as well as in malignancy.

Chapter 5

5. Transforming growth factor (TGF)- β 1 and the Smad pathway in airway EMT.

5.1 Introduction

In this chapter I have focused on TGF β 1 and its canonical ‘SMAD’ pathway cascades (Smad 2/3 and Smad 7), since this is probably the most mentioned generally in the EMT literature. It seemed a sensible place to start in dissecting out potential intracellular EMT mechanisms in the airway context of COPD pathology. Emphasis has been laid both on its expression and also possible functional relationships in terms of airway obstruction in COPD.

TGF β 1, as important multifunctional cytokine involved in number of biological processes including regulation of angiogenesis and ECM components and has been implicated as a driver of COPD airway pathology (Hogg 2004; Kranenburg et al. 2005; Schiller, M., Javelaud, D. & Mauviel, A. 2004). Although TGF β 1 works in association with other pathways as well, the best described intracellular pathway for TGF β 1 actions is the so called “canonical” cascade by involving up-regulation and phosphorylation of Smad 2/3 (Hogg, J. C. & Timens, W. 2009; Schiller, M., Javelaud, D. & Mauviel, A. 2004); that leads to EMT (Xu, J, Lamouille & Derynck 2009), which is active in COPD as shown in chapter 4.

TGF β 1 and its downstream pathway involving different types of Smad transcription factors, including both excitatory and inhibitory Smads, have been reviewed in Chapter 2. To briefly recap, Springer and colleagues previously demonstrated reduced Smad 7 gene expression in bronchial biopsies of COPD patients (Springer et al. 2004), suggesting that lack of Smad 7 could be relevant to COPD pathogenesis (Willis & Borok 2007). Our group have suggested that TGF β 1 is functionally active through pSmad 2/3 expression in COPD (Soltani, Sohal, et al. 2012). The present study has taken this further and evaluated TGF β 1 expression and its associated downstream “SMAD fingerprint” in airway biopsy material from COPD subjects and appropriate smoking and non-smoking controls. This study facilitates the understanding of EMT-associated pathogenesis of COPD as a result of smoking-induced intracellular

downstream Smad transcriptional pathways.

In the present study, I have investigated whether TGF β ₁ and activated (phosphorylated, p) Smad 2/3 expression is increased in smokers, especially in COPD subjects. In addition, reciprocal down regulation of a protective role of pSmad 7 has been investigated. Moreover, I observed a significant relationship between activation of the TGF β ₁-Smad pathway and EMT activity and airflow obstruction.

5.2 Overview of materials and methods

Once again I employed the classical immune-histochemical methods described in chapter 3, to evaluate TGF β ₁, pSmad 2/3 and pSmad 7 expression in the large airway bronchial biopsies collected from well phenotyped volunteer study participants. Detail demographics of subjects are given in Table 5.1.

| Groups (numbers) | NC (n=15) | NLFS (n=15) | S-COPD (n=17) | ES-COPD (n=15) |
|---|-------------------------|--------------------------|---------------------------|---------------------------|
| GOLD I/GOLD II‡ | N/A | N/A | 10/7 | 8/7 |
| Male/female | 7/8 | 11/4 | 9/8 | 9/6 |
| Age (years) | 44 (20-68) (p=0.313) | 50 (30-66) (p=0.313) | 61 (46-78) (p=0.001)* | 62 (53-69) (p=0.001)* |
| Smoking (pack years) | 0 | 32 (10-57) | 45 (18-78) | 51 (18-150) |
| FEV ₁ % predicted (Post BD)† | 113 (86-140) | 99 (78-125) (p=0.01)* | 83 (66-102) (p<0.001)* | 83 (54-104) (p<0.001)* |
| FEV ₁ /FVC % (Post BD)† | 82 (71-88) | 77 (70-96) (p=0.218) | 59 (46-68) (p<0.001)* | 57 (38-68) p<0.001)* |

Table 5.1: Demographic detail and lung function data for participants.

Data expressed as median and range.

NC- Normal control; NLFS-Normal lung function smoker; COPD-CS-COPD current smoker; COPD-ES- COPD ex-smoker; N/A-Not any

*Significance difference from NC

† Post BD values after 400µg of salbutamol

‡ Diagnosis of COPD was made according to GOLD 2015 guidelines

5.3 Results

5.3.1 TGF β ₁ expression

5.3.1.A Basal epithelium

A greater proportion of the cells in the basal layer of the large airway epithelium stained for TGF β ₁ in NLFS, S-COPD and ES-COPD in comparison to H-N control group ($p < 0.05$), but with no significant difference between smoker/ COPD groups (Figure 5.1 and 5.2).

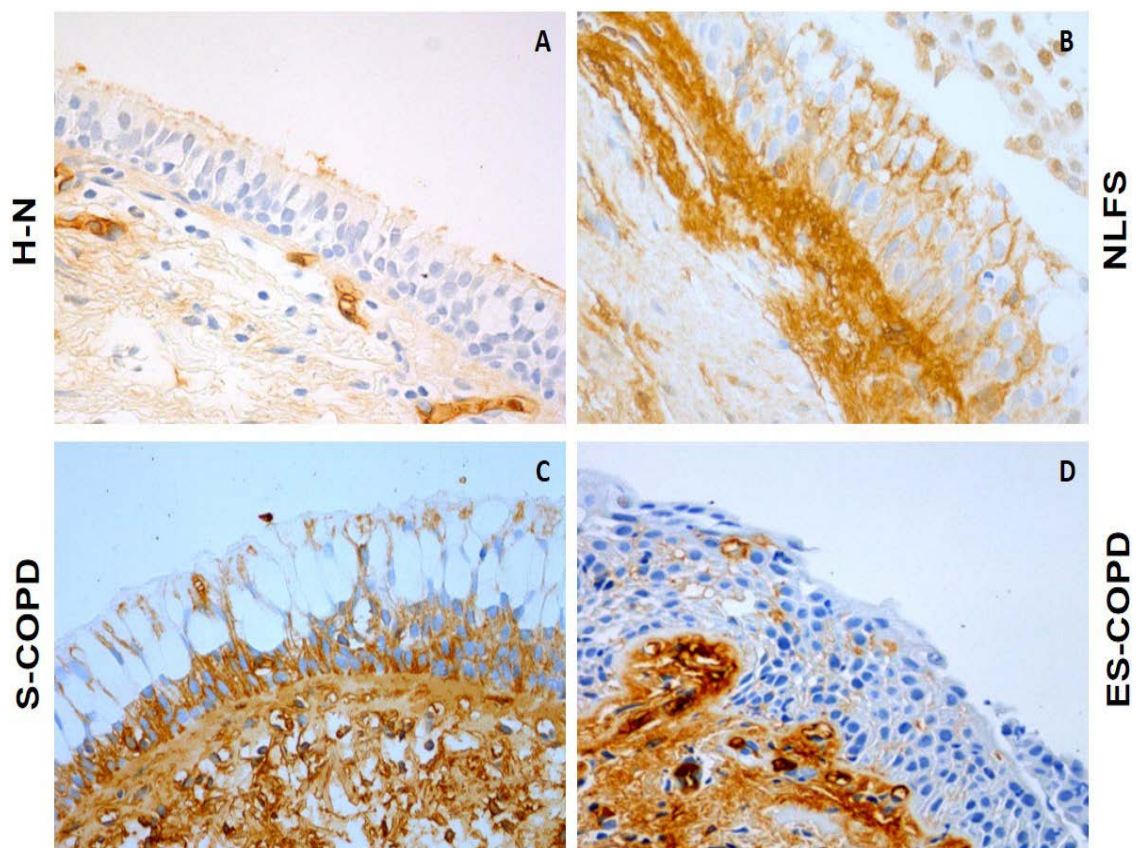


Figure 5-1: Representative photomicrograph of TGF β ₁ expression in 4 different study group.

A) Healthy non-smoker (H-N), B) smoker with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smoker with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .

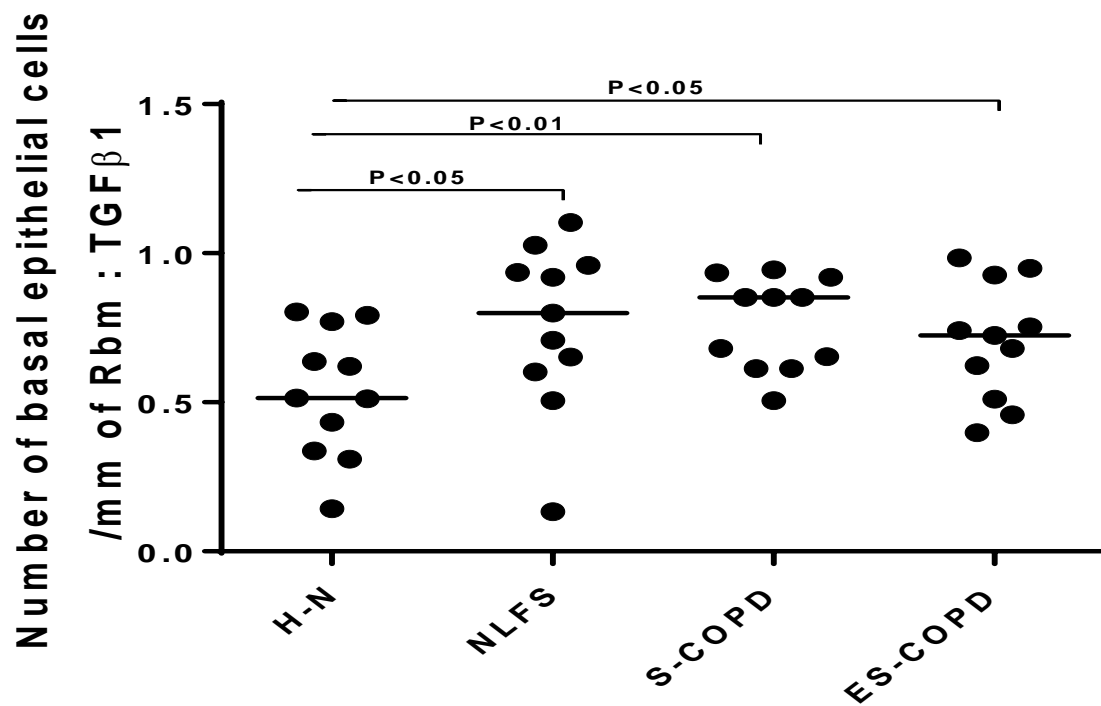


Figure 5-2: Comparison of TGFβ1 expression in basal epithelial cells

between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.1.B Rbm

Rbm cells highly expressed $TGF\beta_1$ in NLFS, S-COPD, and ES-COPD in comparison to H-N control group ($P<0.005$), but this was most marked in S-COPD i.e. there was both a smoking and extra COPD effect ($P<0.005$) (Figure 5.1 and 5.3).

$TGF\beta_1$ expression on blood vessels in the Rbm was significantly increased in all smoker/COPD groups ($p<0.05$) (Figure 5.1 and 5.4). The data were also analysed as percent of vessels that stained, to normalise for a marked increase in smokers/COPD (Soltani, A. et al. 2010), but the signal was much the same.

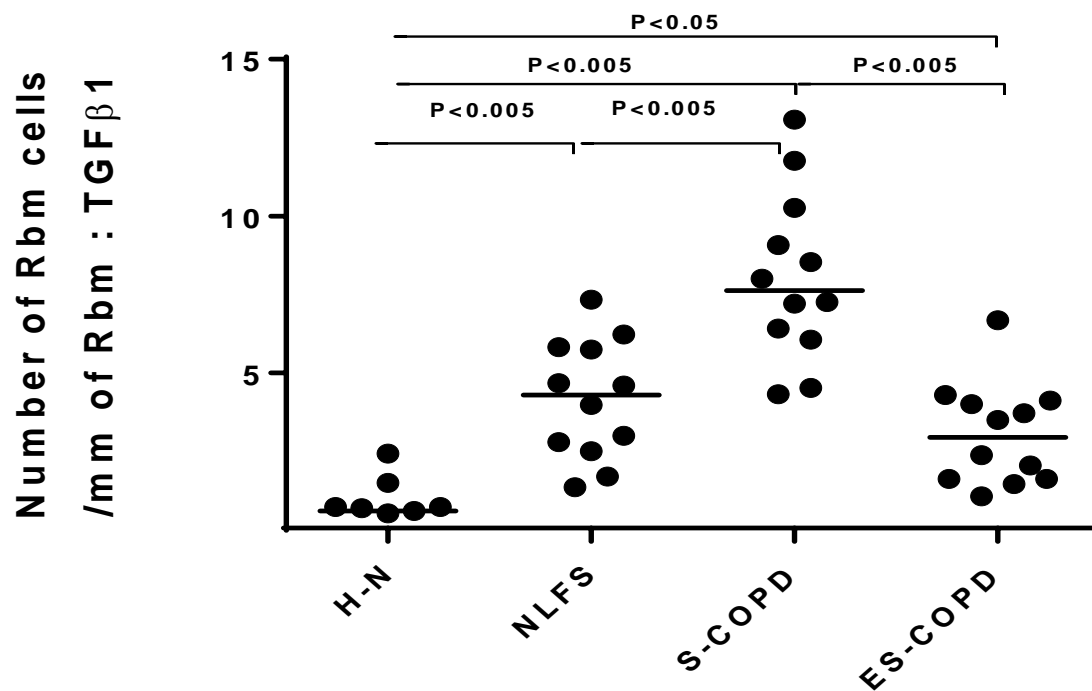


Figure 5-3: Comparison of $TGF\beta_1$ expression in Rbm cells

between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

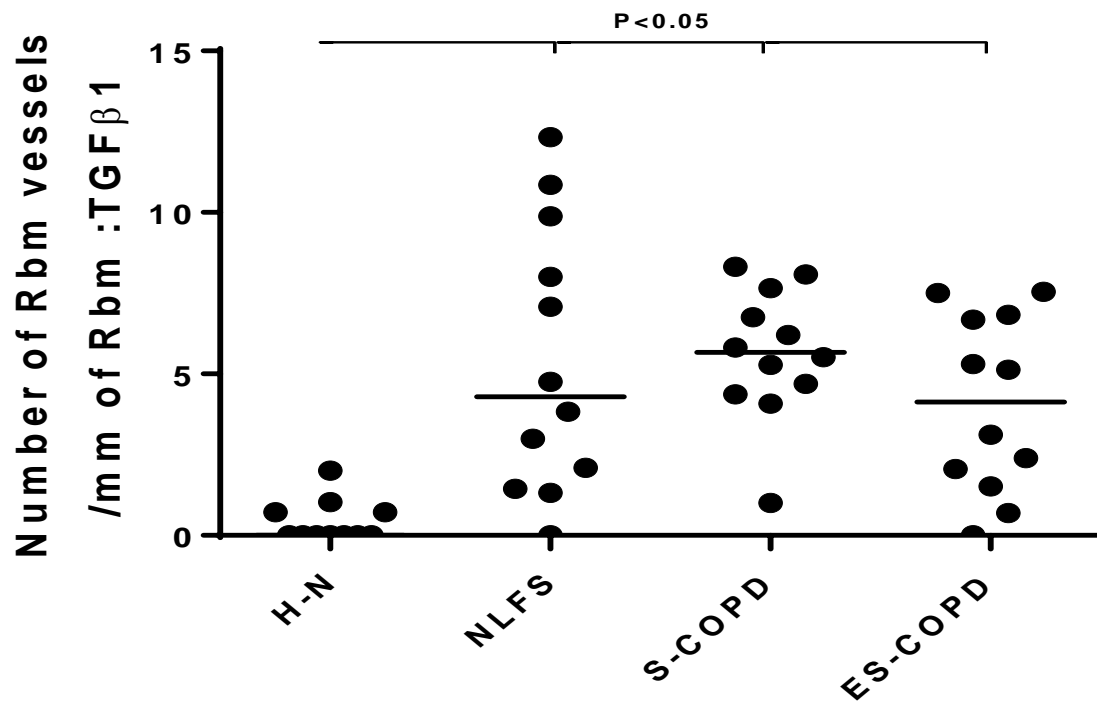


Figure 5-4: Comparison of TGFβ1 expression in Rbm vessels

between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.1.C LP

In the LP, there was either no or just light diffuse staining in the normal controls, which was uniformly much heavier in all smoker/COPD groups (Figure 5.1).

5.3.2 pSmad 2/3 expression

5.3.2.A Basal epithelium

Compared to normal control (H-N), there was an increased proportion of cells in the epithelial basal staining for pSmad 2/3 expression in smoking groups (both NLFS and S-COPD) ($P < 0.05$) but not in the ES-COPD group. (Figure 5.5 and 5.6).

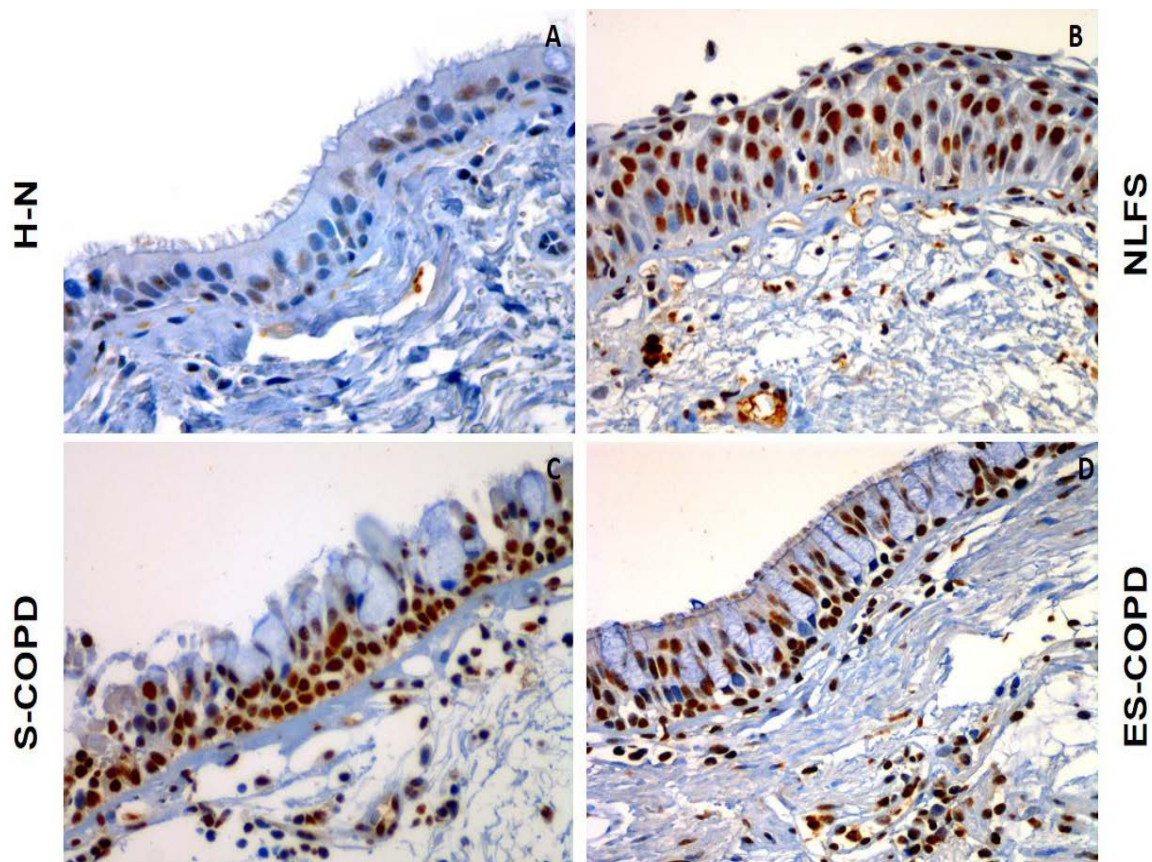


Figure 5-5: Photomicrographs of pSmad 2/3 expression representing 4 different study groups.

A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smokers with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .

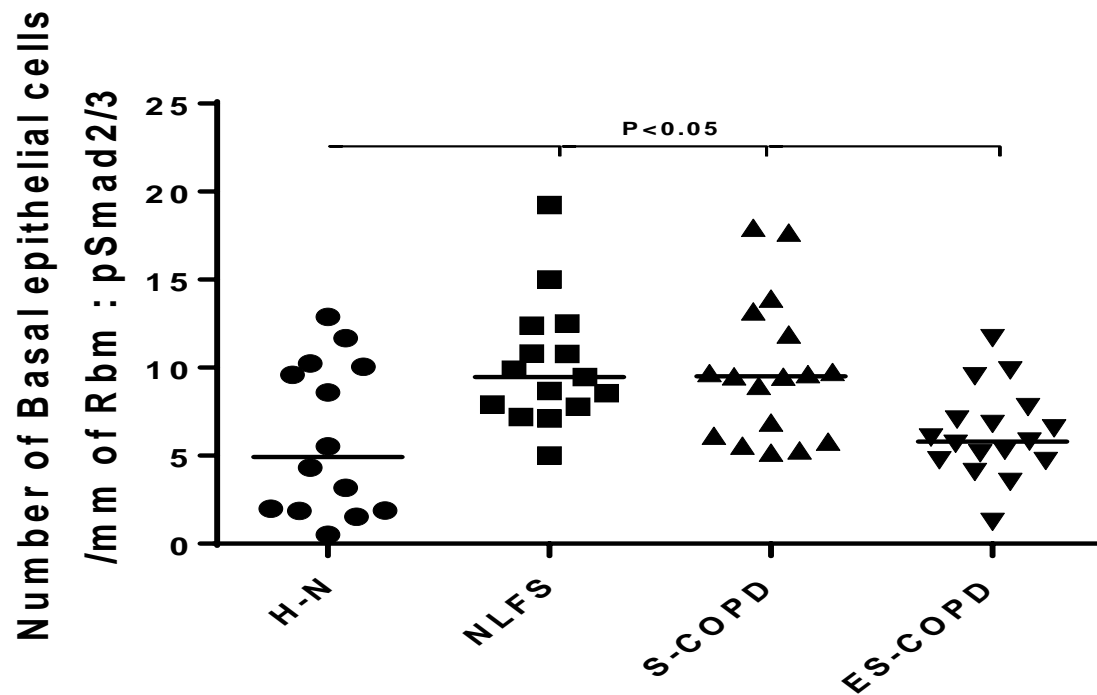


Figure 5-6: Comparison of pSmad 2/3 expression in basal epithelial cells

between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.2.B Rbm

In Rbm cells, an elevated expression of pSmad 2/3 in Rbm cells in the ES-COPD group was intermediate between controls and the other pathological groups. i.e., there was predominantly a smoking effect but again some extra COPD effect (Figure 5.5 and 5.7).

Similarly, pSmad 2/3 Rbm vessel expression was greater in the NLFS and S-COPD groups, but not in the ES-COPD group (Figure 5.5 and 5.8). When normalised as percent vessels, the outstanding feature was a marked increase in staining in the S-COPD group.

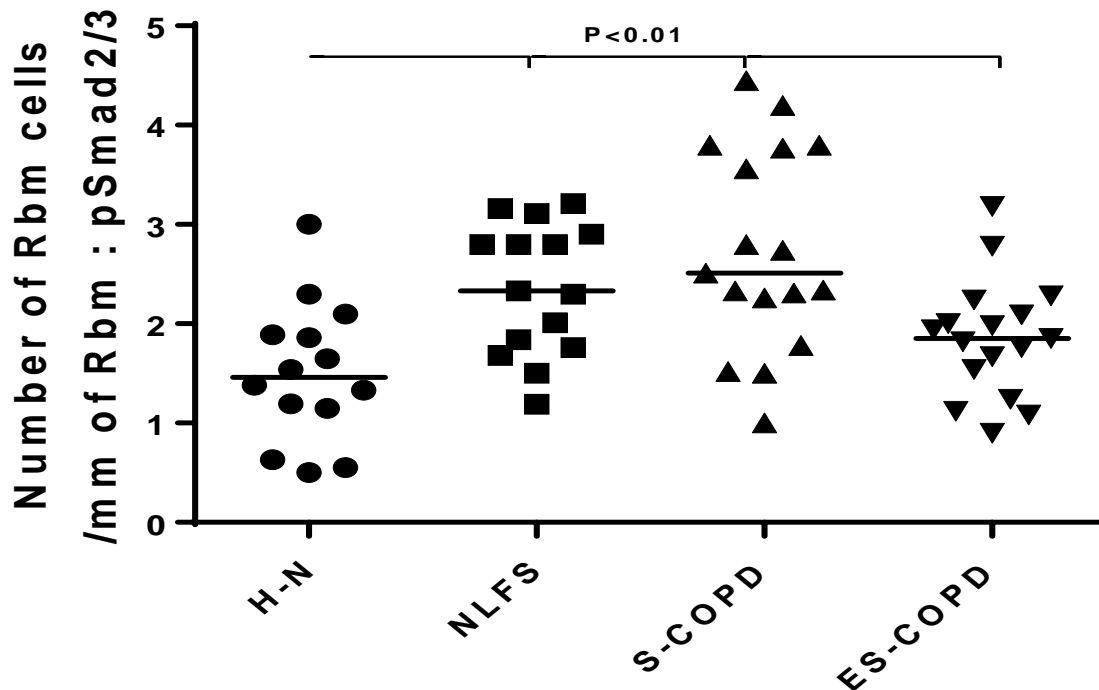


Figure 5-7: Comparison of pSmad 2/3 expression in Rbm cells

between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.2.C LP

In LP cells there was an increase pSmad 2/3 expression only in the COPD groups, perhaps a little more marked in S-COPD ($P < 0.001$) (Figure 5.5 and 5.9) Total cell number in the LP are decreased in COPD, so data were analysed as percentage of cells expressing pSmad 2/3 and was especially marked in the S-COPD group (Figure 5.10). In the LP vessels, there was an increase in pSmad 2/3 expression in both smoking groups, but again especially so in current smoking COPD (S-COPD) ($P < 0.005$) (Figure 5.11).

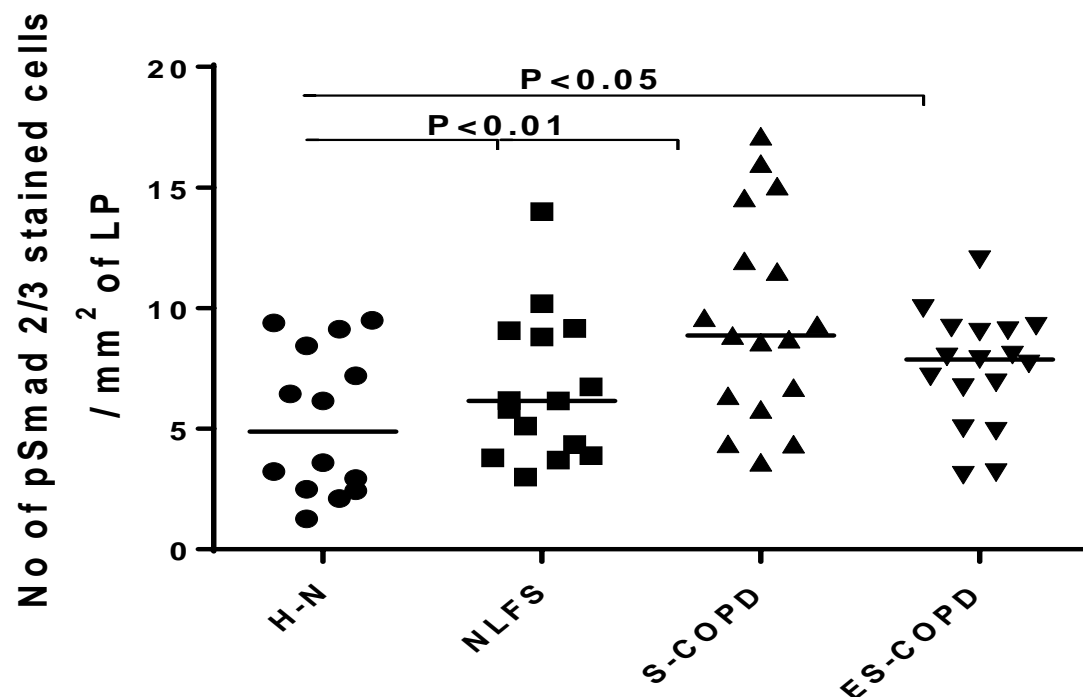


Figure 5-9: Comparison of pSmad 2/3 expression in LP cells

between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

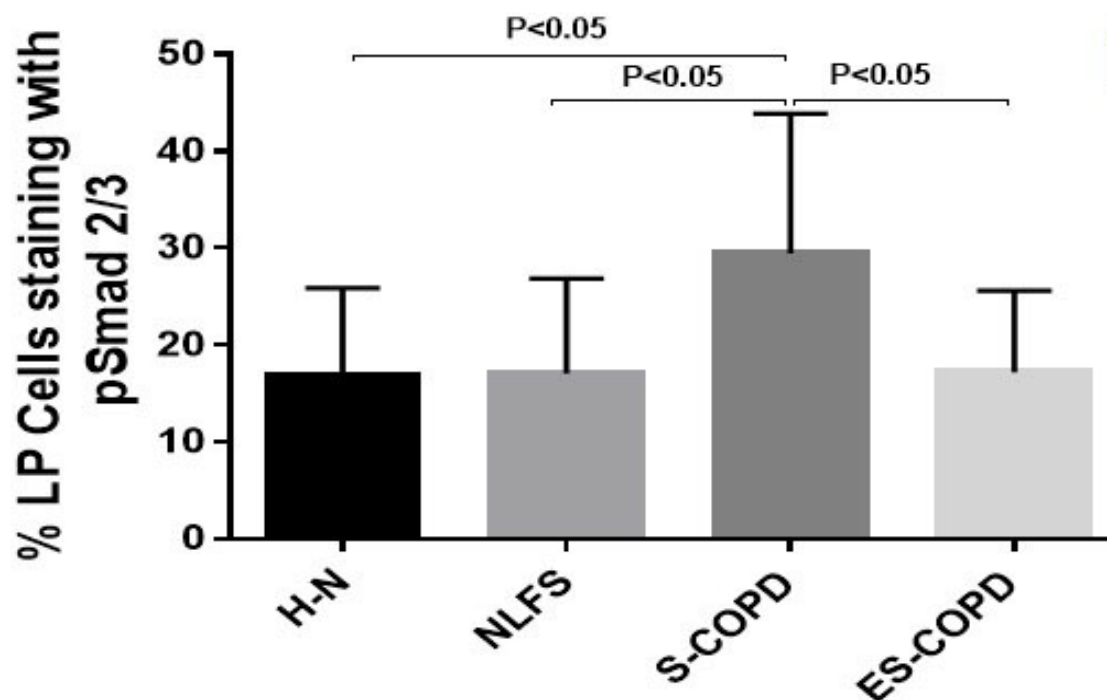


Figure 5-10: Comparison of pSmad 2/3 expression as percent of LP cells between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

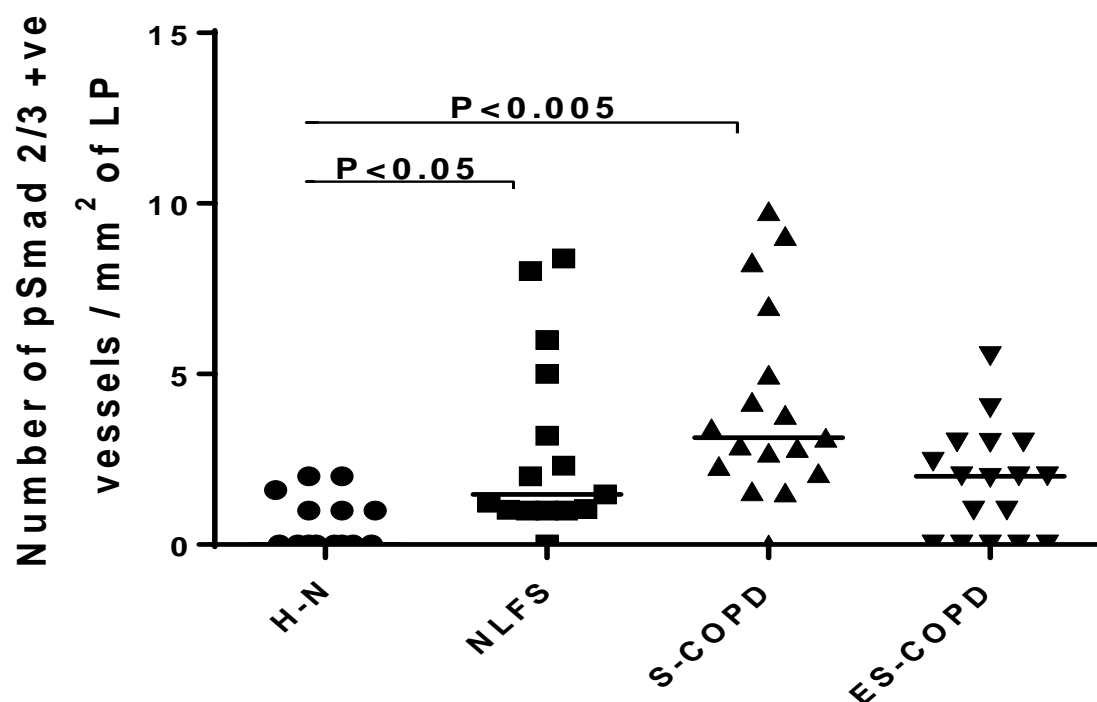


Figure 5-11: Comparison of pSmad 2/3 expression in LP vessels between healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.3 pSmad 7 expression

5.3.3.A Basal epithelium

In general, pSmad 7 expression was inverse of that for pSmad 2/3, but not always.

In contrast to pSmad 2/3, pSmad 7 stained cells in the basal epithelium were slightly increased in NLFS ($P<0.01$) but slightly reduced though non-significantly so in the COPD groups (Figure 5.12 and 5.13).

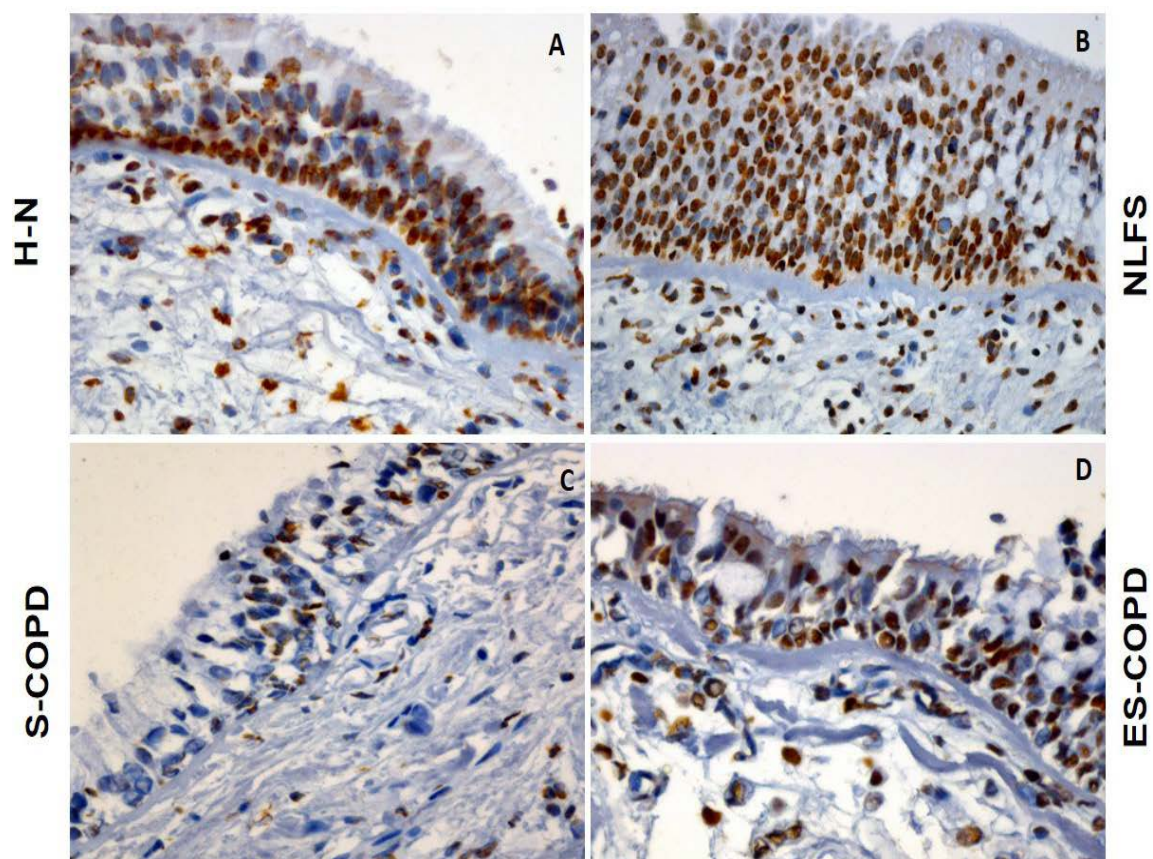


Figure 5-12: Photomicrograph of pSmad 7 expression in 4 different study groups

A) Healthy non-smokers (H-N), B) smokers with normal lung function (NLFS), C) current smoking COPD (S-COPD) and D) ex-smoker with COPD (ES-COPD). Original magnification, $\times 400$. Scale bar = 50 μm .

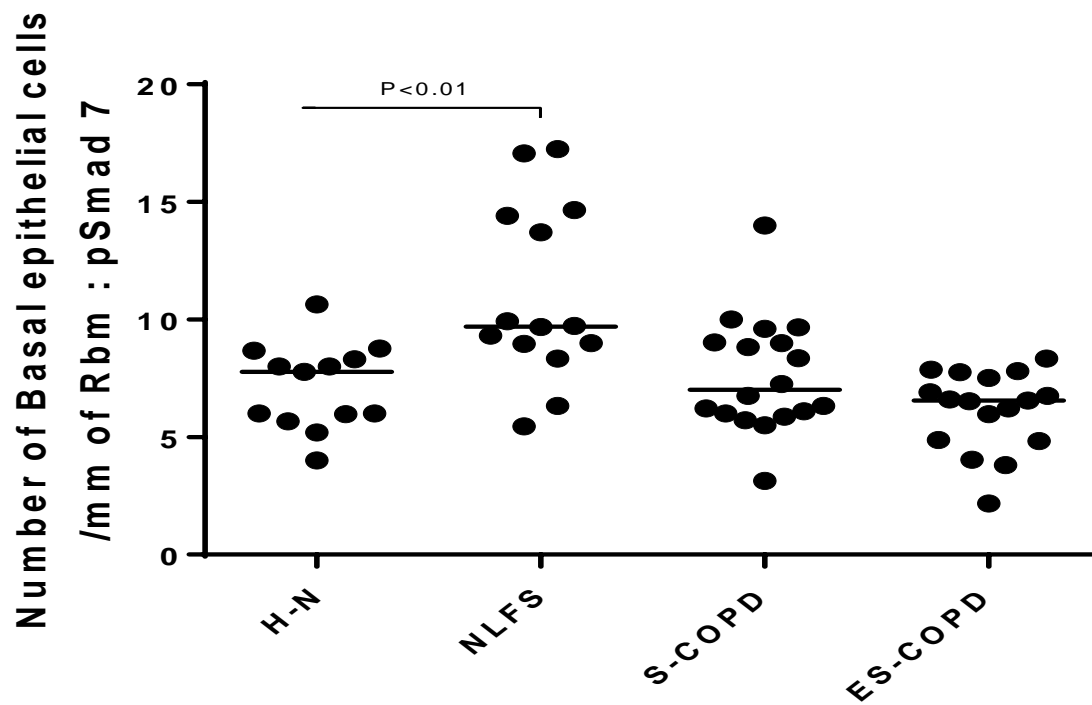


Figure 5-13: Comparison of pSmad 7 expression in basal epithelial cells

In healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

In the Rbm, there was little change in pSmad 7 cell expression, with a slight with a slight decrease only in COPD ($P<0.13$) (Figure 5.12 and Fig 5.14). The pattern in Rbm vessels was similar; though the decrease in percent vessel staining in S-COPD was much more marked ($P<0.01$) (not included here).



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5.3.3.C LP

In LP, there was a reduction in absolute numbers of pSmad 7 cell expression in smokers and COPD about equally ($P<0.01$) (Figure 5.12 and 5.15). In LP vessels, there was a light non-significant decrease in pSmad 7 expression in NLFS which was more marked in both COPD groups ($P<0.05$) (Figure 5.12 and 5.16), and especially then normalised in percentage terms.

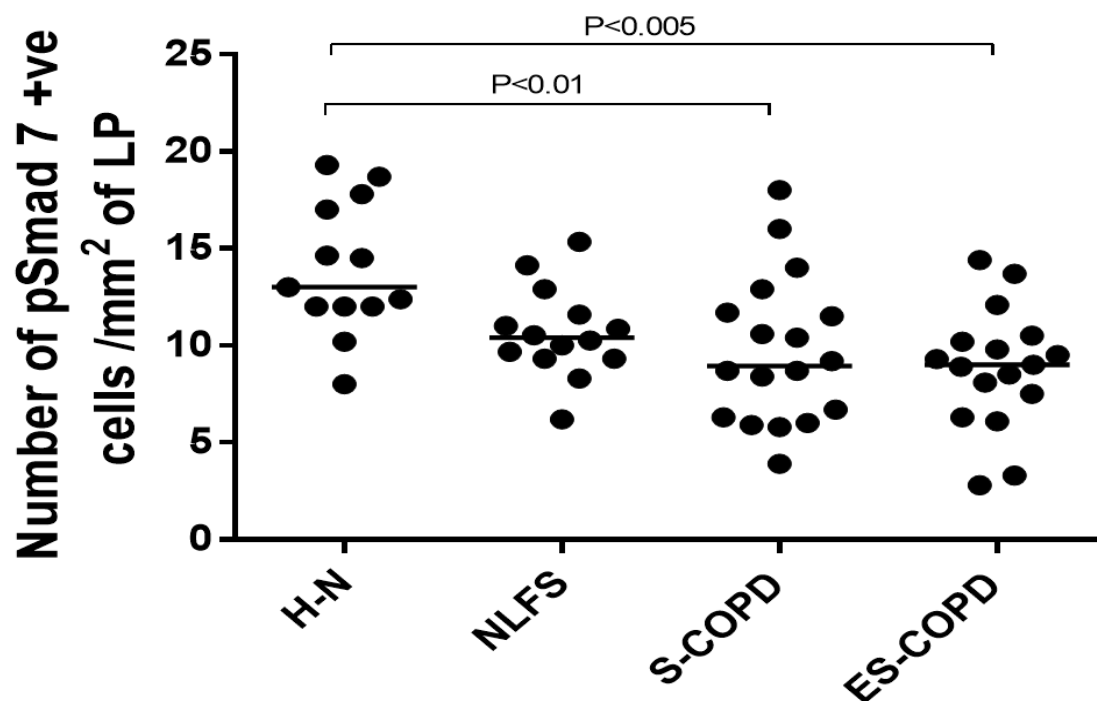


Figure 5-15: Comparison of pSmad 7 expression in LP cells

In healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.4 pSmad 2/3:7 ratios

In the epithelium, there was a significant increase in this ratio only in current smoker COPD (S-COPD) subjects (Figure 5.17). In the Rbm there was a large increase in pSmad 2/3 to 7 ratio in both COPD groups (Figure 5.18).

Finally, in LP cells there was again a marked increase in the ratio in both COPD groups ($P < 0.001$; Figure 5.19).

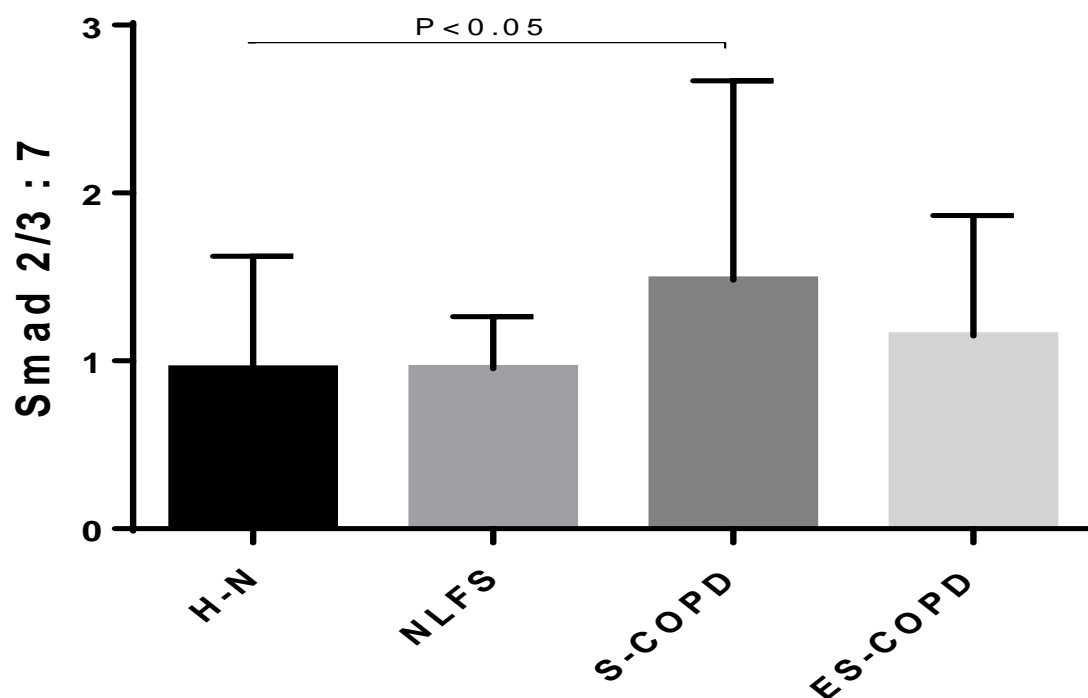


Figure 5-17:: Comparison of cellular expression of pSmad 2/3:7 ratio in basal epithelial cells

of healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

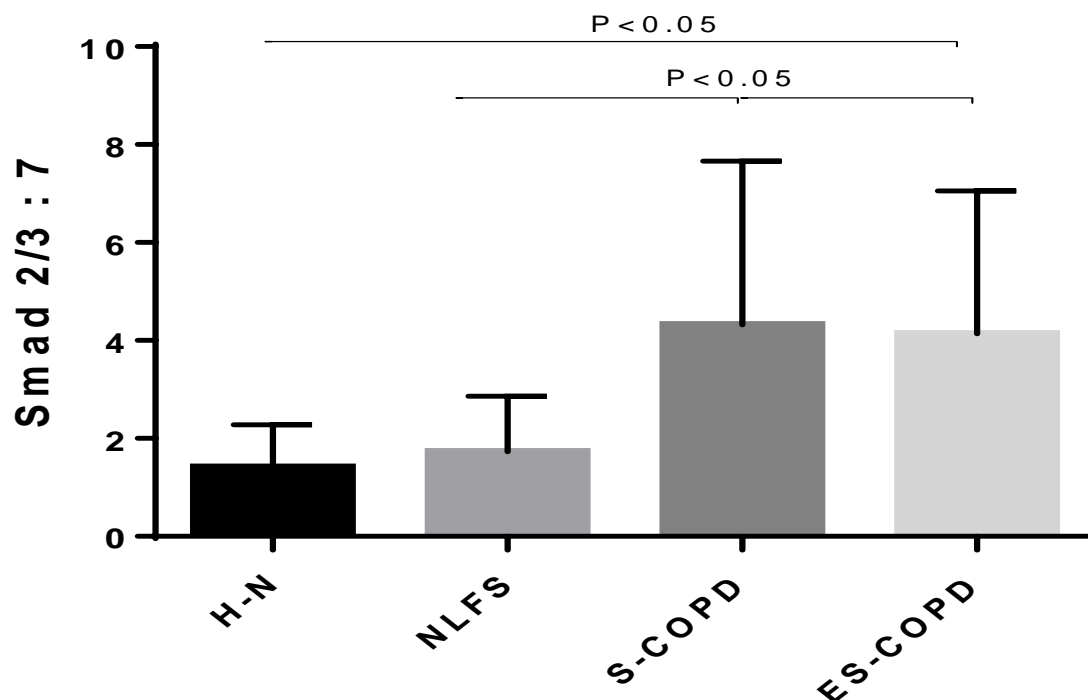


Figure 5-18: Comparison of cellular expression of pSmad 2/3:7 ratio in Rbm cells of healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

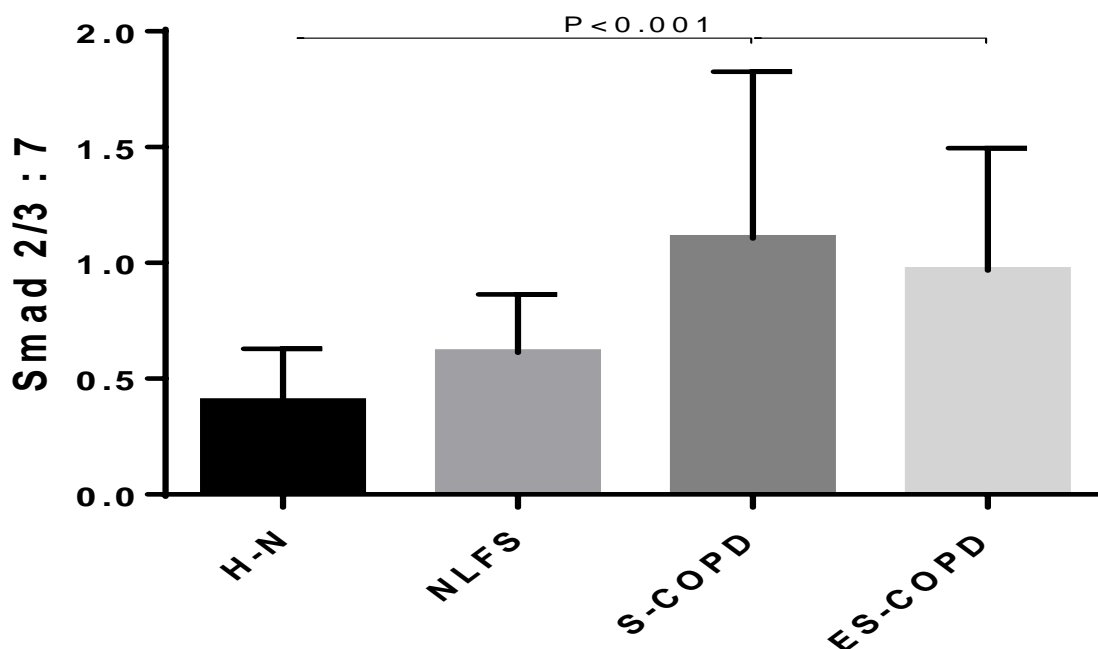


Figure 5-19: Comparison of cellular expression of pSmad 2/3:7 ratio in LP cells of healthy non-smokers (H-N), smokers with normal lung function (NLFS), current smoking COPD (S-COPD) and ex-smokers with COPD (ES-COPD).

5.3.5 Regression analyses

Whether with FEV₁, FEV₁/FVC ratio (FER), or FEF₂₅₋₇₅ % (small airways), there were remarkably consistent negative relationships in all smokers/COPD groups with pSmad 2/3 cell expression (Figure 5.20), i.e. airflow obstruction; and contrasting positive relationships with pSmad 7 expression (Figure 5.21). This is illustrated in ES-COPD, a group less likely confounded by co-existing effects of active smoking, but relationships were all very similar in each non-normal groups.

Cellular expression of both pSmad 2/3 and pSmad 7 were correlated with the classical EMT mesenchymal marker S100A4 (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010); there were consistent relationships between epithelial basal cell and Rbm cell expression of pSmad 2/3 (positive) and pSmad 7 (negative) with S100A4 (Figure 5.22). Similarly, the cellular pSmad 2/3:7 ratios also had consistently positive relationships with the EMT marker.

In contrast, were no significant relationships for basal cell TGFβ₁ expression against pSmads or S100A4, nor with lung function.

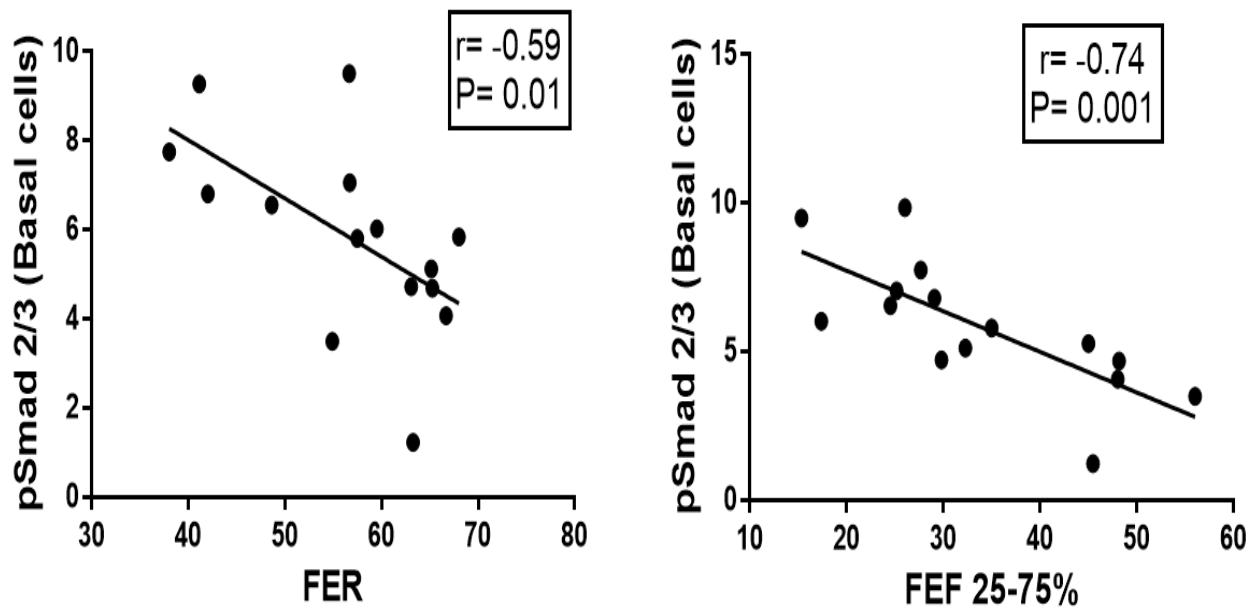


Figure 5-20: Correlation between basal epithelial pSmad 2/3 expression and lung function test

for the ex-smoker with COPD group (ES-COPD).

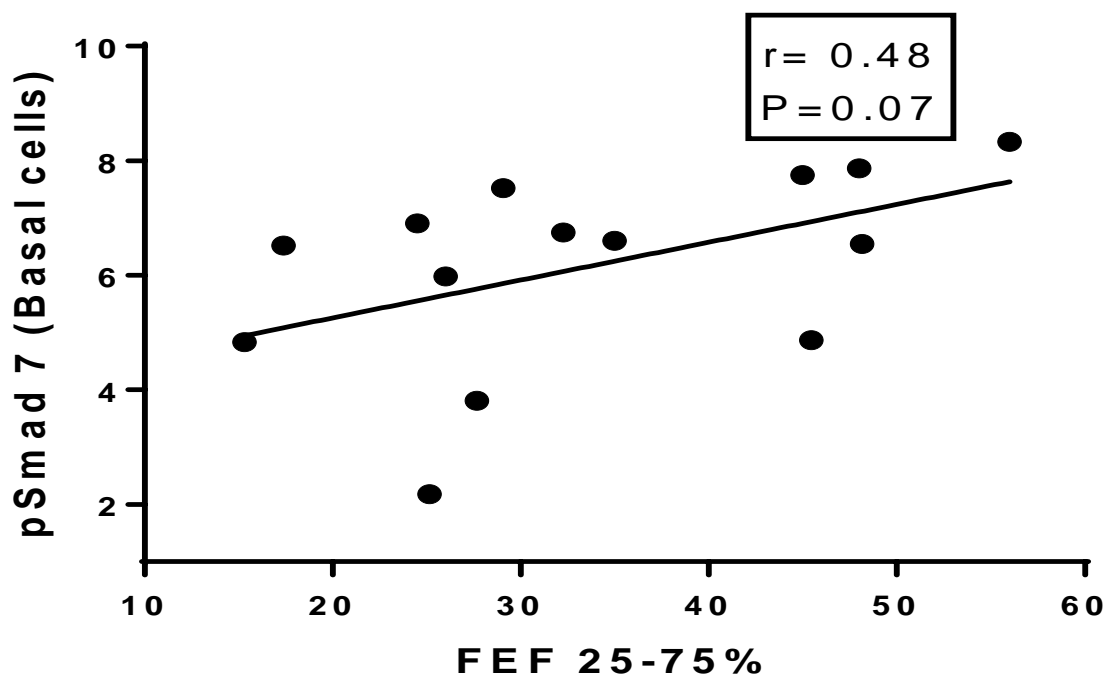


Figure 5-21: Correlation between basal epithelial cells pSmad 7 expression and FEF 25-75 %

for the ex-smoker with COPD group (ES-COPD).

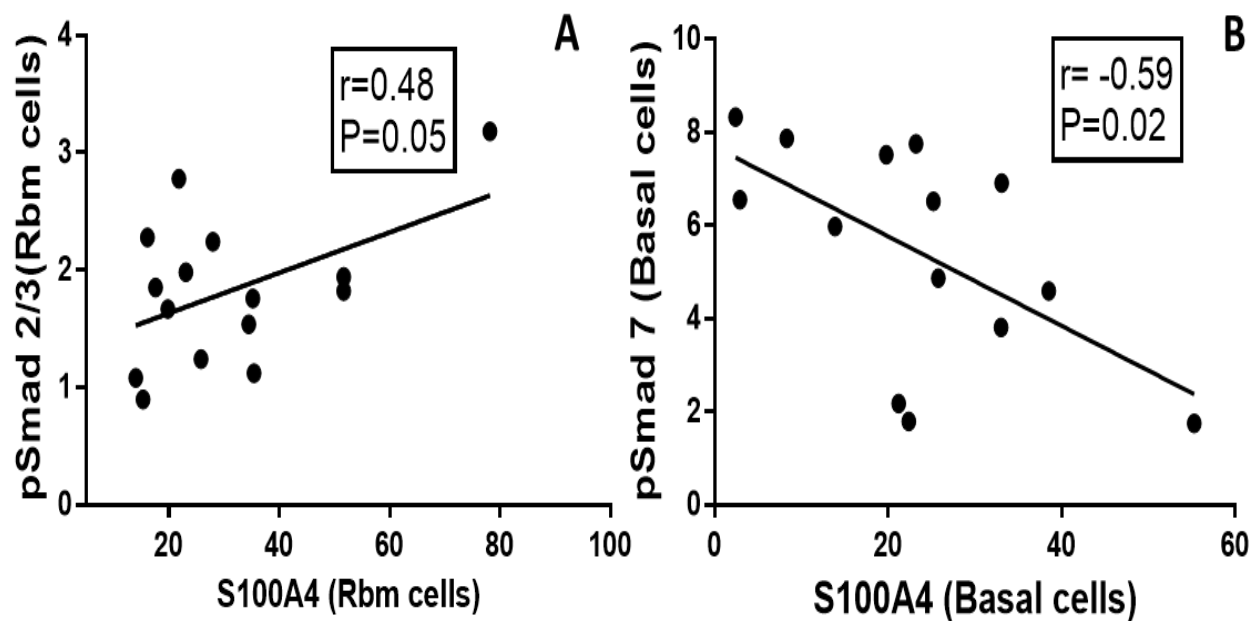


Figure 5-22: Correlation between S100A4, pSmad 2/3 and pSmad 7 expression

(A) Correlation between numbers of Rbm cells positive for pSmad 2/3 with S100A4 positive Rbm cells; (B) Correlation between numbers of basal epithelial cells positive for pSmad 7 with S100A4 positive basal cells, for the ex-smoker with COPD group (ES-COPD).

5.4 Discussion

As mentioned earlier, the basic pathophysiology of COPD lies in its progressive fibrosis and destruction of small airways (Hogg, McDonough & Suzuki 2013), though there has been little understanding of this. However, there is increasing evidence of dysregulated epithelial basal stem cell function (Ryan et al. 2014), one feature of which our group strongly suggested is active EMT (Sohal, S. S., Reid, Soltani, Ward, Weston & Muller 2010). The literature around this is limited and reviewed earlier.

TGF β ₁ has been suggested as a key regulator of the fibrotic airway disease in COPD (Tatler & Jenkins 2012), but again there is little confirmation of this. Although cigarette smoke was shown to induce TGF β ₁ in cultured mouse tracheal explants (Wang, R. D., Wright, J. L. & Churg, A. 2005), its down-stream effectors, pSmad 2/3, were reported not to be increased in expression in small airways in COPD subjects (Zandvoort et al. 2006) though Smad 7 expression was reduced (Zandvoort et al. 2006). TGF β ₁ has been postulated also as a key inducer of EMT in several tissues, including alveolar type II cells (Konigshoff et al. 2009) in idiopathic pulmonary fibrosis (IPF) (Ramos et al. 2001), so some connection between TGF β ₁, EMT and fibrosis is appearing in lung pathology.

This is the first comprehensive study of the expression of the TGF β ₁-related “classical” pathway transcriptional proteins in airway tissues from normal smokers and patients with COPD compared to normal controls. I have studied this in large airways because our group previously reported TGF β ₁ expression to be increased in the epithelial and Rbm cells of smokers and especially those with COPD (Soltani, Sohal, et al. 2012) but focused only on the relationship of TGF β ₁ to vascular structures.

The observation of an increase in TGF β ₁ expression in basal airway epithelial cells in smokers is consistent with some previous reports in both large and small airway (de Boer et al. 1998; Takizawa et al. 2001). In the current study the fact that cellular pSmad expression was closely associated in regressions with both EMT activity and airflow obstruction suggest strongly that this pathway is of functional significance in COPD pathogenesis.

Several genetic studies have demonstrated a link between COPD and TGF β ₁ in COPD (Smolonska et al. 2009). TGF β ₁ can also induce EMT- type changes in cultured respiratory epithelial cells (Gohy, S. T. et al. 2015). The epithelium in smokers is also likely to produce more TGF- β ₁, suggesting the possibility of a self-reinforcing vicious cycle to EMT stimulation (Wang, R. D., Wright, J. L. & Churg, A. 2005). My finding is novel in that I have shown both a general increase in TGF β ₁ in all airway wall compartments studied, but a parallel change in downstream pSmad signalling. These changes were evident in both smokers and COPD, but most in actively smoking COPD. The relevance of my findings is underscored by anti-TGF β treatment being able to attenuate COPD pathology in mice (Podowski et al. 2012).

In my work, although TGF β ₁ expression was increased in the smoker/COPD airways, unlike for pSmad there was no relationship to either EMT activity or obstructive lung function. This suggests that other growth factors or changes in receptor responsiveness to TGF β ₁ are also involved. Further, the Wnt- β catenin pathway may be a mediator in pulmonary fibrosis (Konigshoff et al. 2008) and Notch, NF κ B and Hedgehog (Hh) have been also shown to participate in EMT (Karhadkar et al. 2004). Work on these pathways in COPD is now needed, and some contribution to this is provided in next Chapter.

Evidence of increased TGF β ₁ expression by Rbm vessels was further supported by vascular expression of pSmad2/3 in smokers and COPD. This system may be a driving factor for angiogenesis in the Rbm to produce the picture of pre-malignant Type-3 EMT (Kalluri, R. & Weinberg 2009). Such a link with cancer is reinforced by the finding that EMT expression through a Smad-binding element is active in non-small cell lung carcinoma (Yang, H et al. 2015) and facilitates metastasis (Yu et al. 2015). It needs to be noted that lung cancer is especially prevalent in smokers with COPD (Yang, IA et al. 2011)

The large majority of cells in the LP are thought to be stromal (Mani et al. 2008) and are likely to be the one expressing pSmads. This would fit with evidence that lung fibroblasts in COPD patients produce increased extracellular matrix (ECM), dependent on the local cytokine environment (Zandvoort et al. 2008).

One of the most remarkable translational findings in my current study was the significant correlation of pSmad 2/3 and pSmad 7 expression with both an EMT activity

marker and airflow obstruction. Interestingly, in more advanced disease and in small airways, Zandvoort and colleagues did not find pSmad 2 expression in COPD (Zandvoort et al. 2006); but it may be that my study being into earlier disease in COPD was more appropriate for investigating core pathogenic mechanisms.

The strengths of the present study include, first, the use of relevant human tissue, with sufficiently robust number, following up my previous observation of presence of EMT in both small and large airways of COPD (chapter 4). Second, by using well phenotyped individuals with mild to moderate COPD, I studied pathogenic mechanisms in relatively early disease without confounding by chronic infection and emphysema. There are also a few limitations to this study. Firstly, it is cross-sectional at a single time point and longitudinal studies are needed to see how variable TGF β ₁-pSmad expression is and if it relates to the trajectory of airway dysfunction. Secondly, I am not yet sure about the true phenotype of the LP cells expressing pSmad which is a future goal now of our group. Finally, my control subjects were somewhat younger than the smoker/COPD group, but there was no evidence that growth factor or transcription factor expression were age-related across any group.

In conclusion, the TGF β ₁-pSmad pathway is likely to be an important regulator of EMT and physiological obstruction, presumably via fibrosis. The present study demonstrated a positive association between smoking, COPD and up regulation of stimulatory pSmads in all airway compartments, and in vessels as well as cells. The relationships seen between pSmad expression and EMT and also airflow obstruction support these statements. Translational research in this area still hold a considerable potential to explore new therapeutic targets in order to revolutionized the management of smoking-related airflow limitation in patients with COPD.

Chapter 6

6. **β -catenin, Twist and Snail: Transcriptional regulation of EMT in smokers and COPD, and relation to airflow obstruction**

6.1 Introduction

Relatively little has been known regarding transcriptional regulation of EMT specifically in the airways of smokers and COPD, although there is a large literature in other pathological situations and especially in epithelial cancers. There is just some work in epithelial cell culture on the mechanisms of EMT in the airways (Gohy, S. T. et al. 2015). In chapter 5, I investigated the evidence for involvement of TGF- β 1 and its SMAD transcription factor system in EMT activity in large airway biopsies from smokers and patients with COPD, and in this chapter I deal with another key question regarding the role of a well described EMT-related transcriptional factor cluster (β -catenin, Snail1 and Twist) specifically in development and progression of airway remodelling. This has not been previously done.

A review of these transcription factor clusters were given in Chapter 2, as well as mutual interaction potentially existing between them.

To my knowledge, it is probably the first study, where not only β -catenin, Snail1 and Twist protein expression has been observed but also cellular compartmentalisation has also been identified in airway biopsy material obtained directly from human COPD subjects and appropriate smoking and non-smoking controls. I also investigated relationships between these transcriptional factors and EMT activity (represented by the mesenchymal marker S100A4), with the TGF β 1 and Smad system, and finally with airflow obstruction as the final functional outcome of these complex processes.

6.2 Overview of materials and methods

I employed classical immune-histochemical methods to evaluate β -catenin, Snail1 and Twist expression in the large airway epithelium in bronchial biopsies collected from well phenotyped study participants. Details of study participants are given in Table 6.1.

| Groups (numbers) | NC (n=15) | NLFS (n=15) | COPD-CS (n=20) | COPD-EX (n=15) |
|---|-------------------------|--------------------------|---------------------------|---------------------------|
| GOLD I/GOLD II‡ | N/A | N/A | 11/9 | 8/7 |
| Male/female | 7/8 | 11/4 | 12/8 | 9/6 |
| Age (years) | 44 (20-68) (p=0.313) | 50 (30-66) (p=0.313) | 60 (48-78) (p=0.001)* | 62 (53-69) (p=0.001)* |
| Smoking (pack years) | 0 | 32 (10-57) | 45 (18-80) | 51 (18-150) |
| FEV ₁ % predicted (Post BD)† | 113 (86-140) | 99 (78-125) (p=0.01)* | 85 (68-102) (p<0.001)* | 83 (54-104) (p<0.001)* |
| FEV ₁ /FVC % (Post BD)† | 82 (71-88) | 77 (70-96) (p=0.218) | 59 (48-68) (p<0.001)* | 57 (38-68) p<0.001)* |

Table 6.1: Demographic detail and lung function data for participants.

Data expressed as median and range.

NC- Normal control; NLFS-Normal lung function smoker; COPD-CS-COPD current smoker; COPD-ES- COPD ex-smoker; N/A-Not any

*Significance difference from NC

† Post BD values after 400µg of salbutamol

‡ Diagnosis of COPD was made according to GOLD 2015 guidelines.

6.3 Results

6.3.1 Basal epithelial cells

6.3.1.A β -catenin

There was general staining for β -catenin in the basal cells of airway epithelium in all groups with no significant difference in number of cells expressing it. However, there were striking change in the cellular distribution of β -catenin expression from cell membrane to cytoplasm in NLFS and COPD-EX, but to the nucleus in COPD-CS (Figure 6.1 and 6.2). The ratio of number of cells with predominant nuclear rather than membranous/cytoplasmic predominance was significantly higher in COPD-CS in comparison to NC and NLFS as well ($P<0.01$) (Figure 6.3).

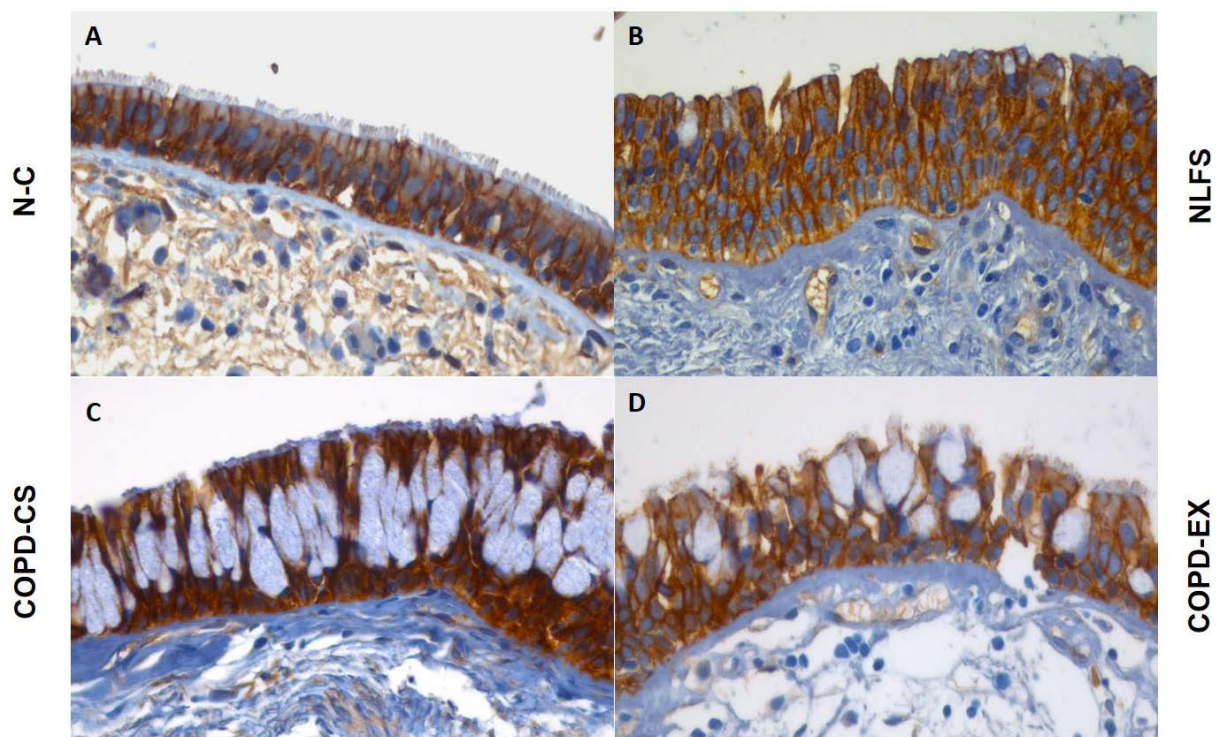


Figure 6-1: Representative photomicrograph of β -catenin expression in 4 different study group.

A) Healthy non-smoker (N-C), B) smoker with normal lung function (NLFS), C) current smoking COPD (COPD-CS) and D) ex-smoker with COPD (COPD-EX). Original magnification, $\times 400$. Scale bar = $50\ \mu\text{m}$.

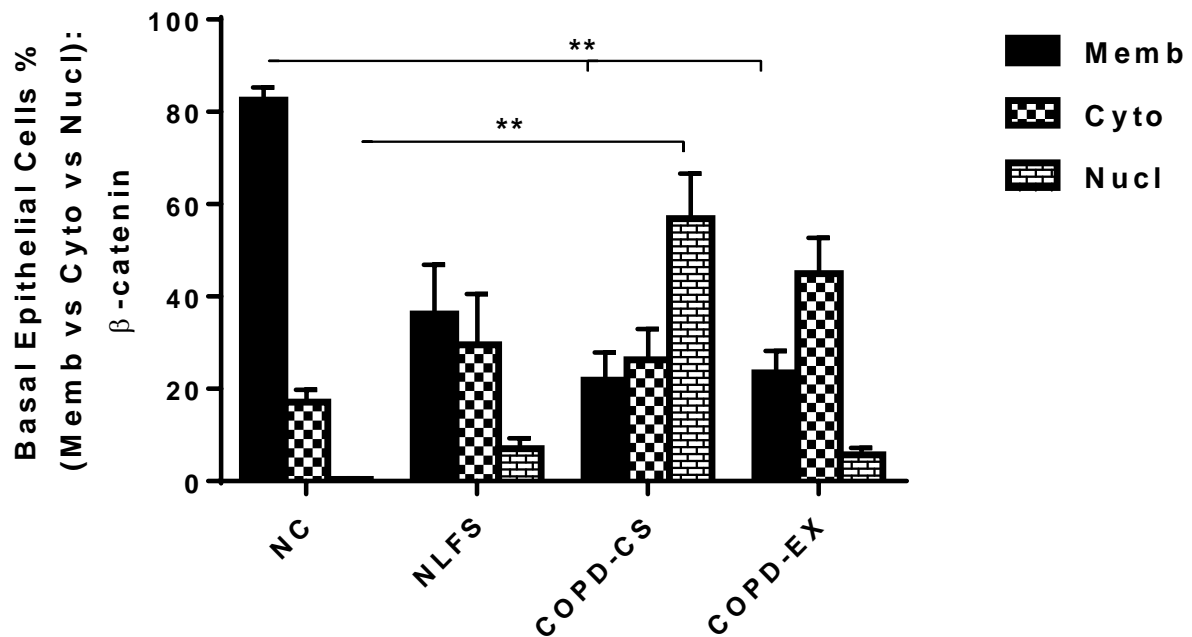


Figure 6-2: Comparison of cytoplasmic versus nuclear % basal cells expressing β -catenin

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

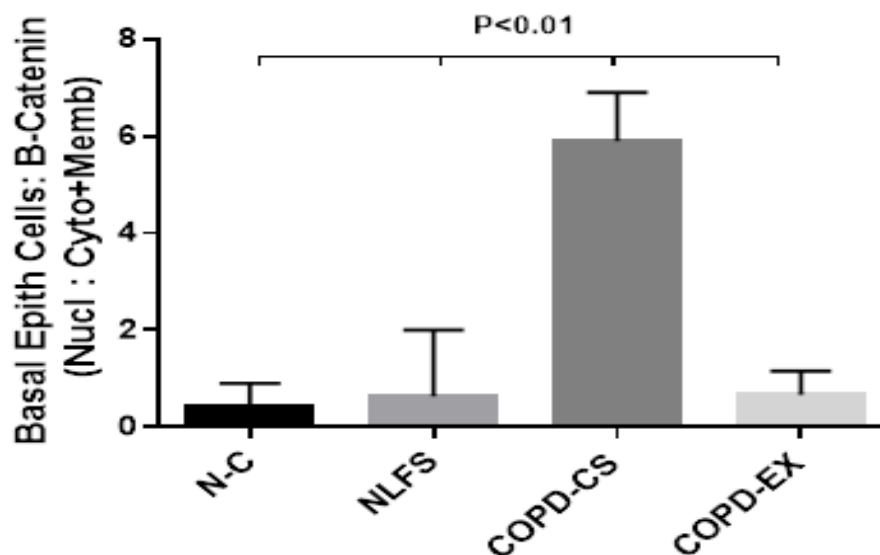


Figure 6-3: Comparison of nuclear: membranous/cytoplasmic ratio of basal cells expressing β -catenin

Between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.1.B Snail1

Snail expression was less uniform among basal cells, but there was a significant increase expression in both NLFS and COPD-CS ($P<0.05$). In addition, there was a significant shift from cytoplasmic to nuclear expression only in COPD-CS group ($P<0.01$) gradually rise from NC to NLFS and finally to the COPD-CS group ($P<0.01$). (Figure 6.4 and 6.5).

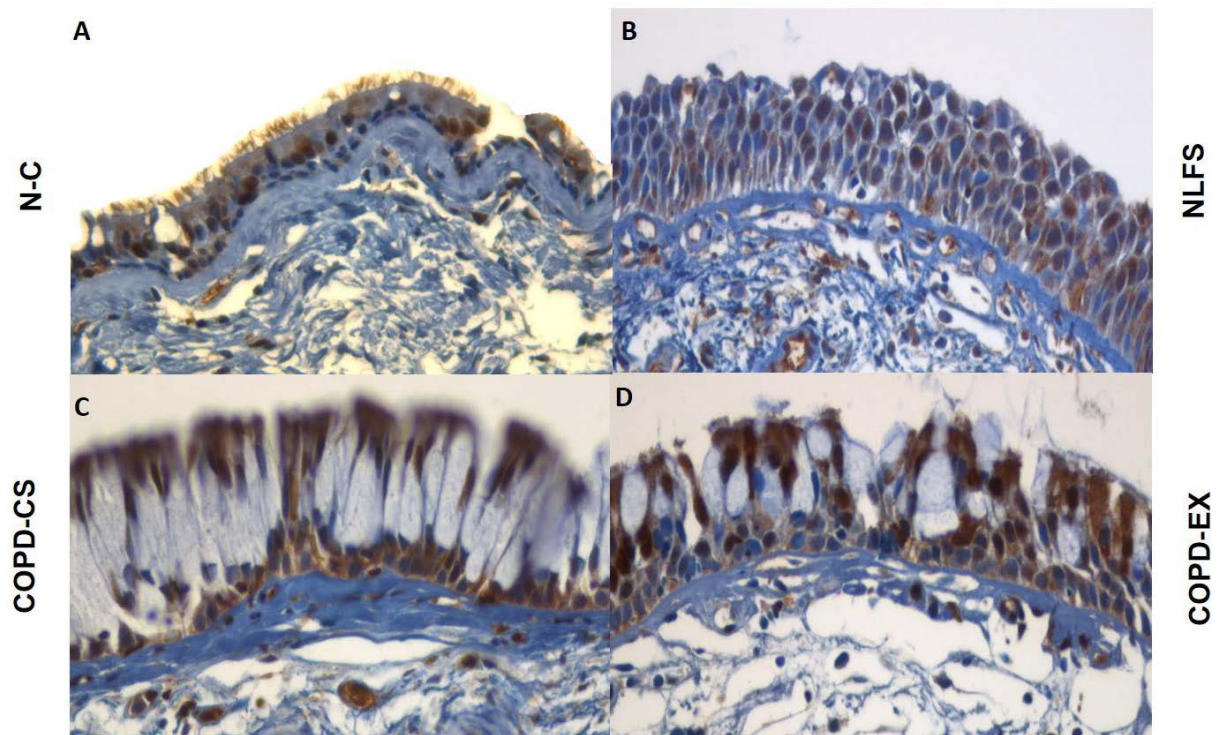


Figure 6-4: Photomicrographs of Snail1 expression representing 4 different study groups:

A) Healthy non-smoker (N-C), B) smoker with normal lung function (NLFS), C) current smoking COPD (COPD-CS) and D) ex-smoker with COPD (COPD-EX). Original magnification, $\times 400$. Scale bar = $50\ \mu\text{m}$.

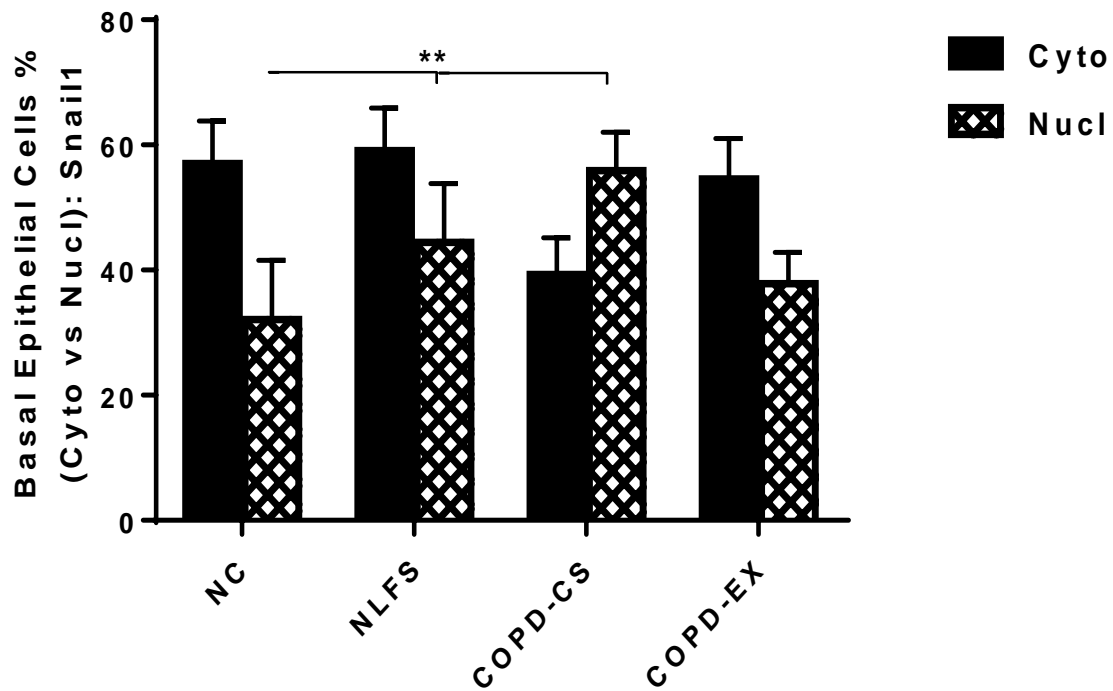


Figure 6-5: Comparison of cytoplasmic versus nuclear % basal cells expressing Snail1

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.1.C Twist

Twist expression was prominent in the NC group but only in the more apical cell areas. In contrast there was more basal cell expression in all other groups ($P < 0.01$). There was shift from the cytoplasmic to nuclear compartment expression only in the 2 groups, NLFS and COPD-EX ($P < 0.05$), and not in COPD-CS (Figure 6.6 and 6.7).

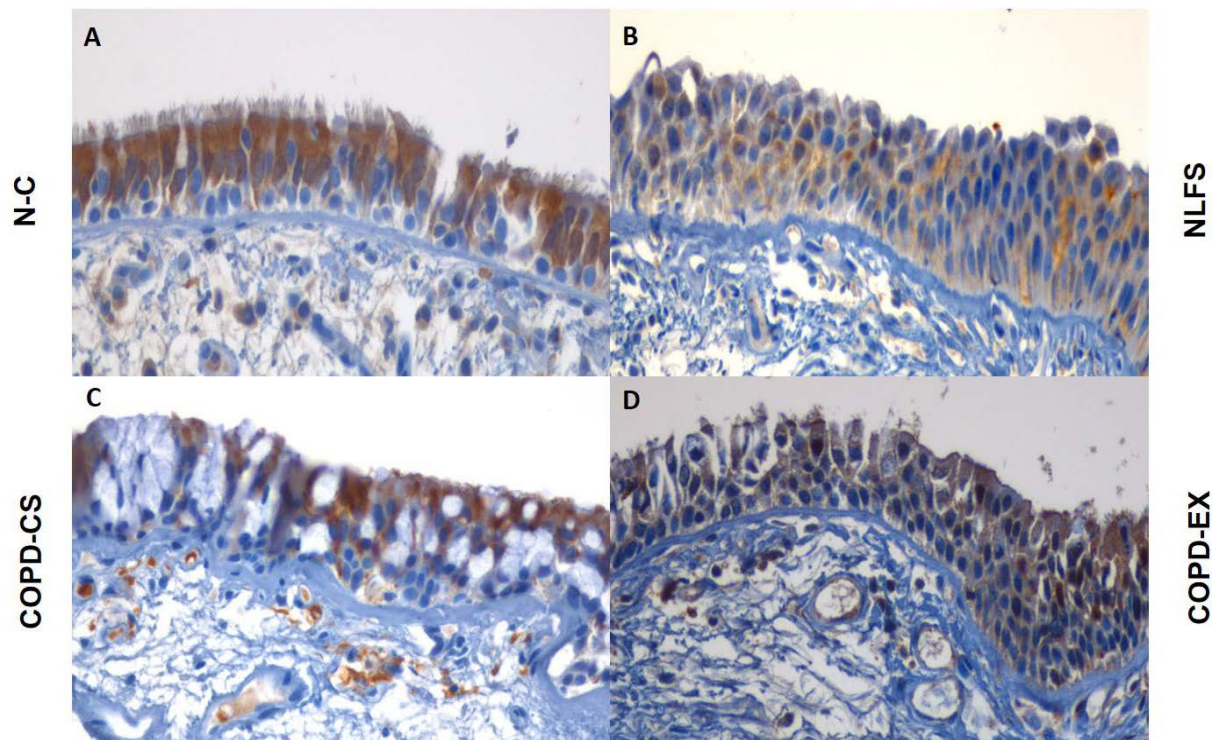


Figure 6-6: Representative photomicrograph of Twist expression in 4 different study groups

A) Healthy non-smoker (N-C), B) smoker with normal lung function (NLFS), C) current smoking COPD (COPD-CS) and D) ex-smoker with COPD (COPD-EX). Original magnification, $\times 400$. Scale bar = $50 \mu\text{m}$.

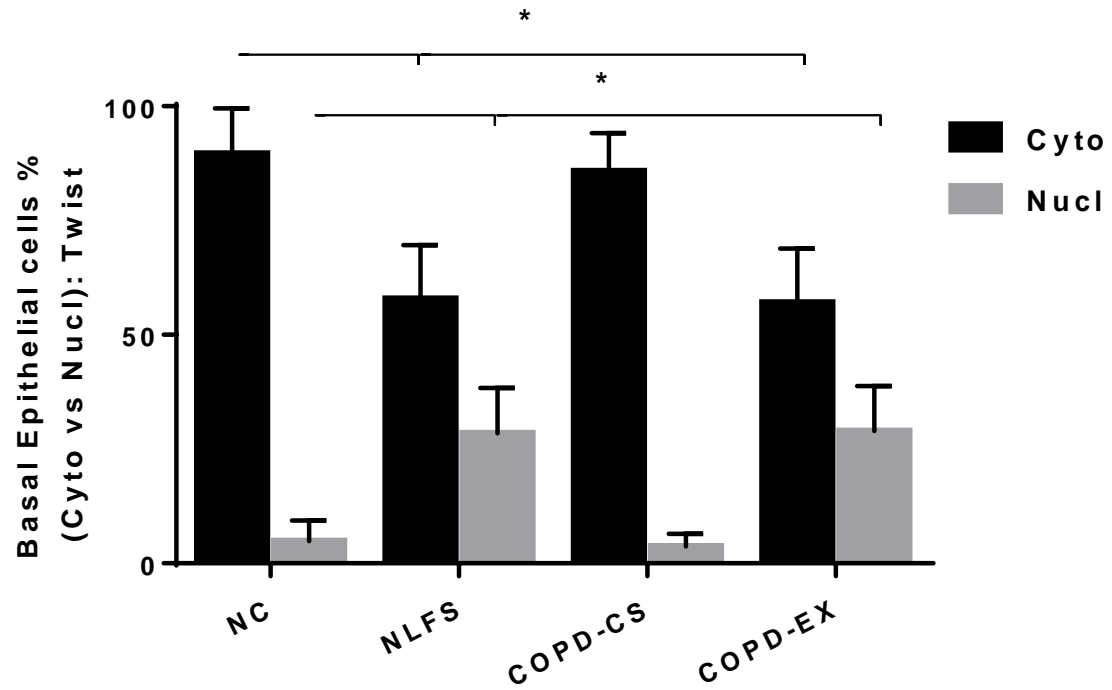


Figure 6-7: Comparison of cytoplasmic versus nuclear % basal cells expressing Twist

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.2 Rbm cells

6.3.2.A β -catenin

There was little difference in general cellular staining between the 4 groups, but a small though significant shift from cytoplasmic to nuclear expression in NLFS and COPD-CS ($P<0.05$) (Figure 6.1 and 6.8). Similarly, nuclear to cytoplasmic ratio was significantly higher than NC in NLFS group ($P<0.05$) (Figure 6.9).

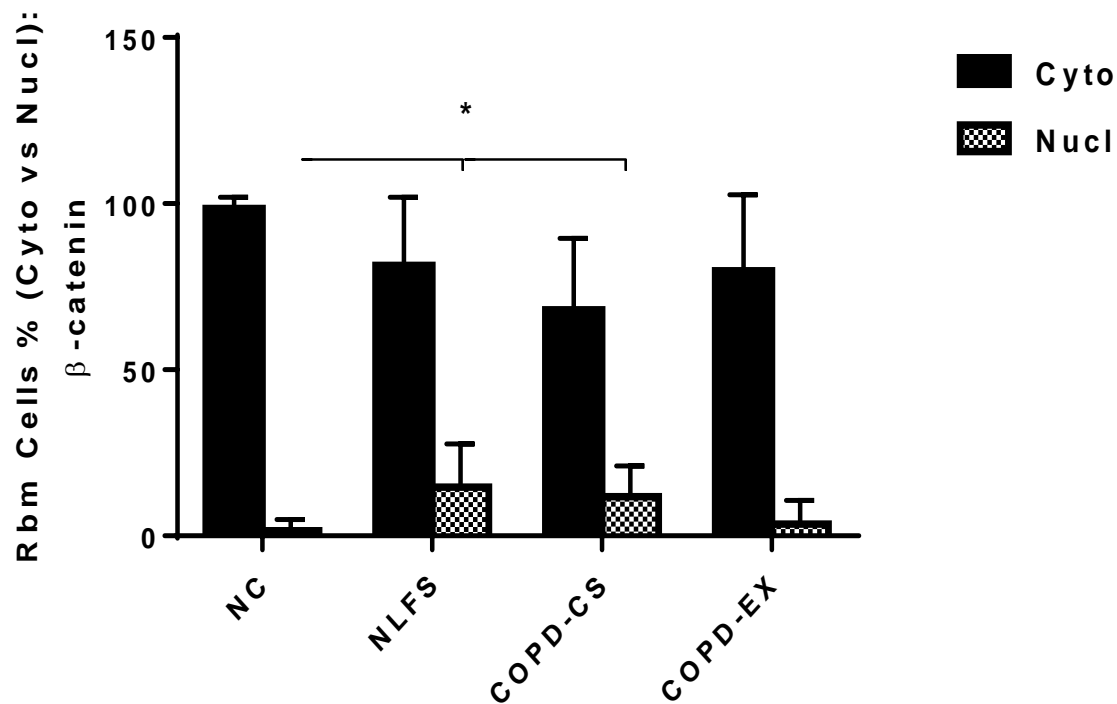


Figure 6-8: Comparison of cytoplasmic versus nuclear % Rbm cells expressing β -catenin

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

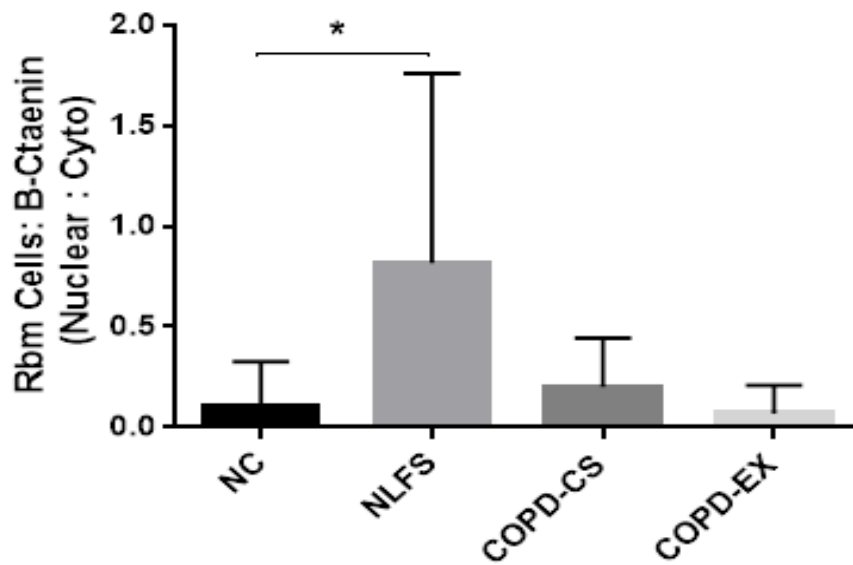


Figure 6-9: Comparison of nuclear: cytoplasmic ratio of Rbm cells expressing β -catenin

In healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.2.B Snail1

There was similar and substantial staining of Snail1 in Rbm cells in all 4 groups, but with a marked shift from cytoplasmic to nuclear expression in the 3 different clinical groups compared to NC ($P<0.05$) (Figure 6.4 and 6.10), reflected in increased nuclear to cytoplasmic ratio for these 3 pathological groups ($P<0.05$).

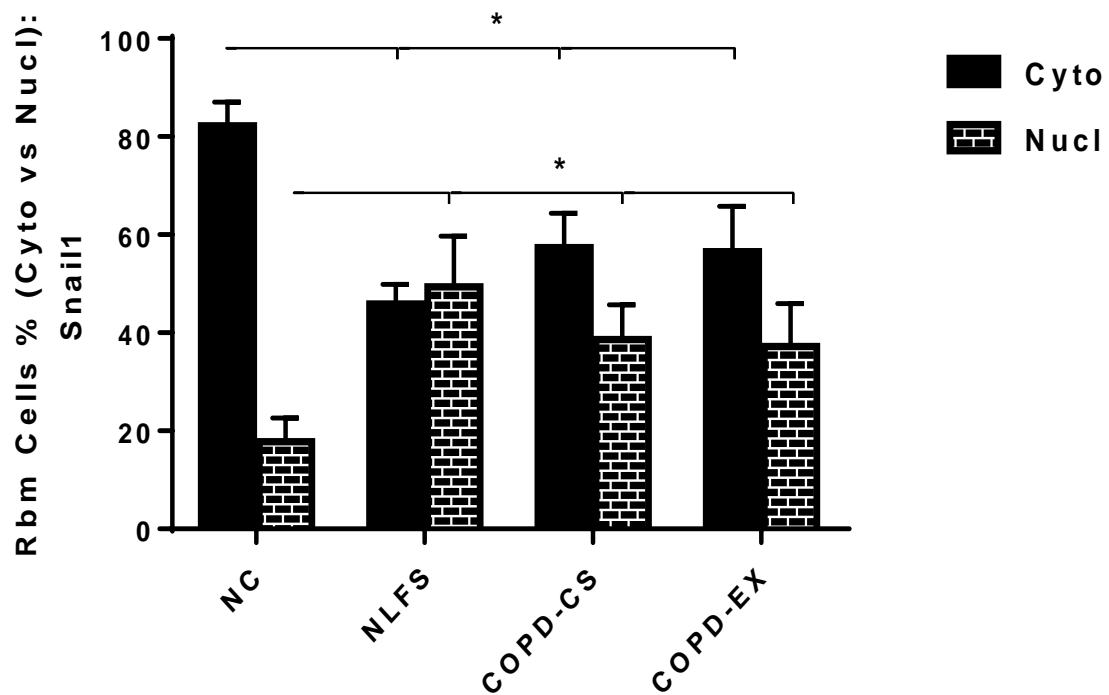


Figure 6-10: Comparison of cytoplasmic versus nuclear % Rbm cells expressing Snail1

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.2.C Twist

There was increased expression of Twist in NLFS as compared NC subjects but a slight shift towards nuclear expression occurred in both NLFS and COPD-EX group ($P<0.05$) (Figure 6.6 and 6.11). A significantly elevated nuclear to cytoplasmic ratio occurred in NLFS and COPD-EX groups ($P<0.01$).

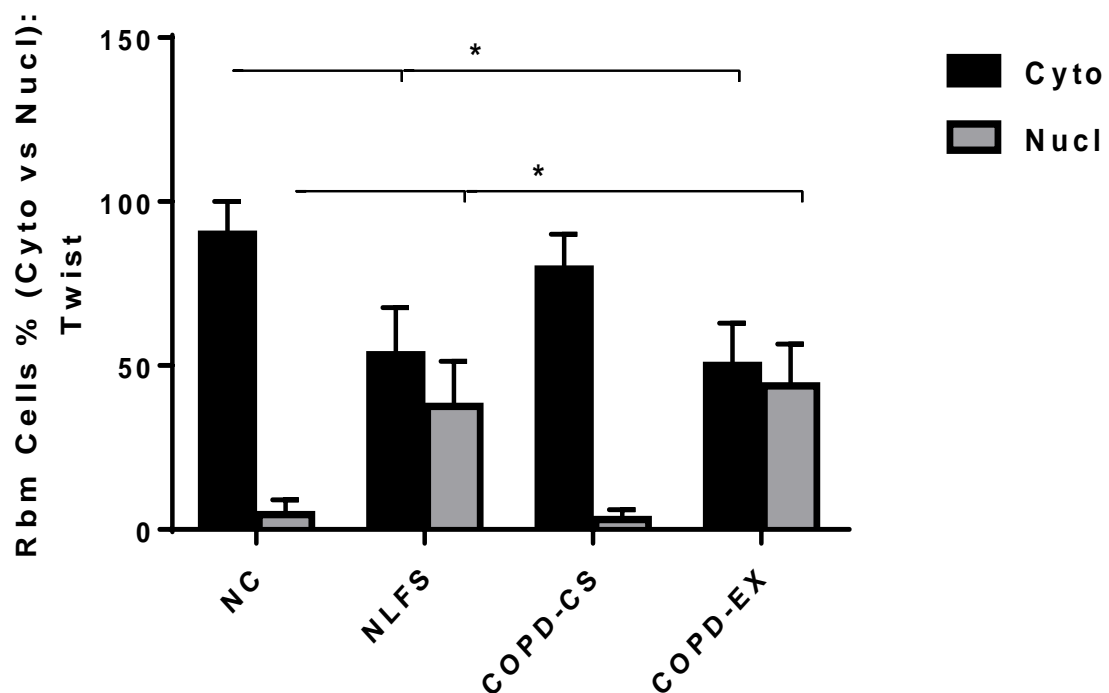


Figure 6-11: Comparison of cytoplasmic versus nuclear % Rbm cells expressing Twist

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.3 LP cells

6.3.3.A β -catenin

Matrix staining for β -catenin was observed in NC only, and was absent in all clinical groups, suggesting some change in physio-chemical interactions with matrix proteins. In LP cells there were fewer cells expressing β -catenin in all groups (approx. 20% of total cells), but there was a shift to nuclear expression in both NLFS and COPD-CS compared to NC ($P < 0.05$). (Figure 6.1 and 6.12) Similarly, nuclear to cytoplasmic ratio was also significantly higher in NLFS and COPD-CS ($P < 0.05$).

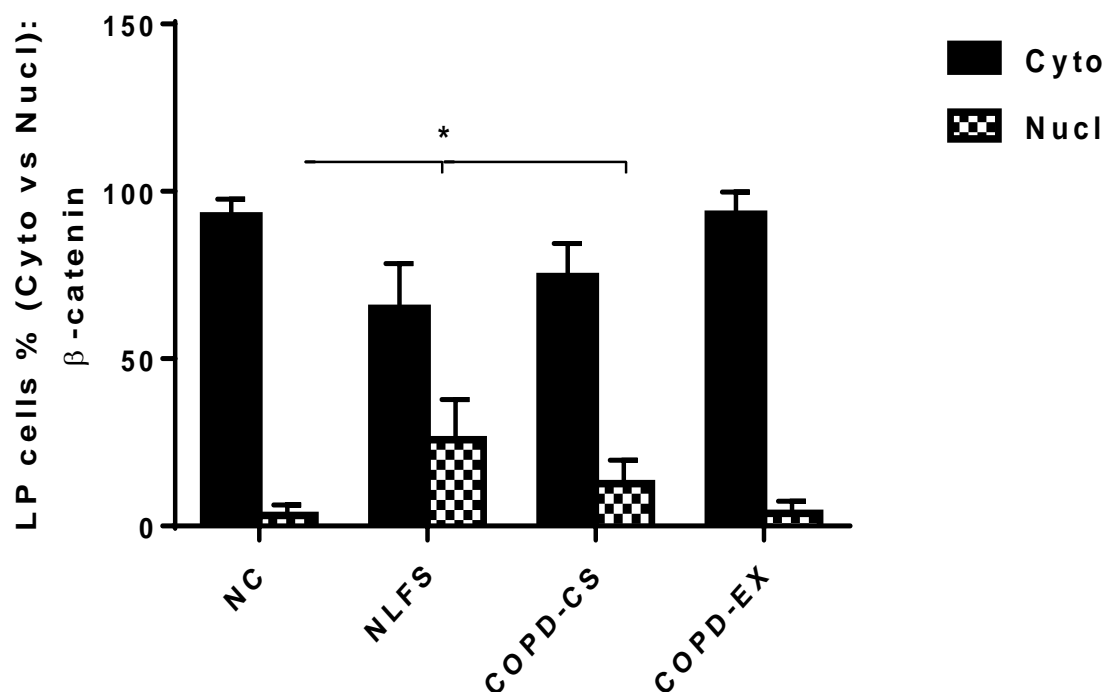


Figure 6-12: Comparison of cytoplasmic versus nuclear % LP cells stained with β -catenin

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.3.B Snail1

No matrix staining was observed in any group. There was again a marked shift from cytoplasmic to nuclear expression in the 3 clinical groups, but especially in NLFS and COPD-CS ($P<0.05$) (Figure 6.4 and 6.13). A corresponding shift was observed in nuclear to cytoplasmic ratio in NLFS and COPD-CS ($P<0.05$).

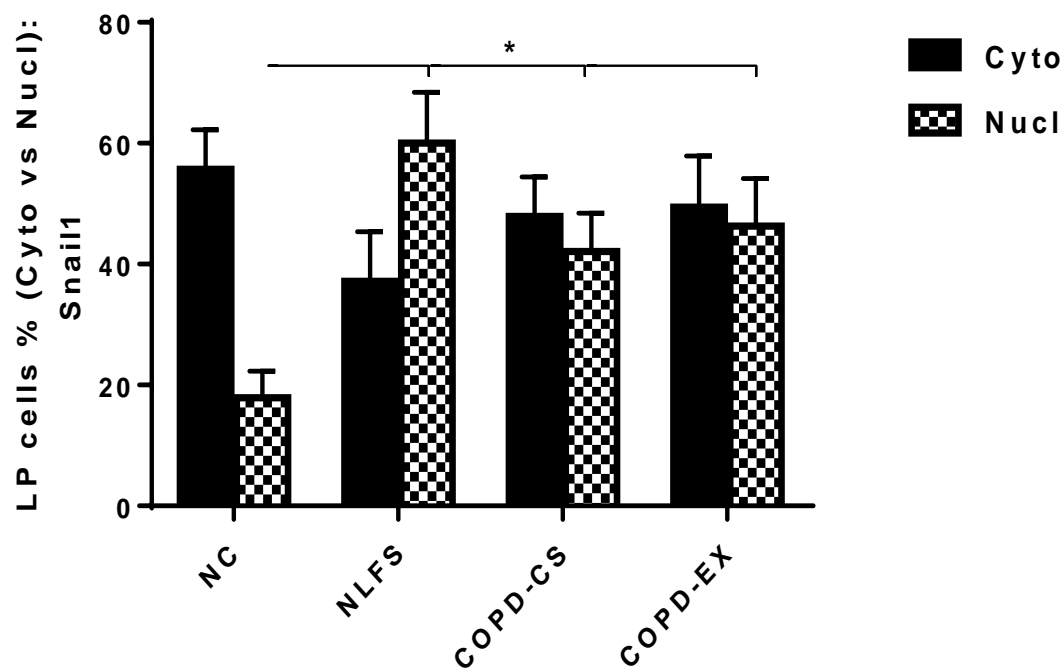


Figure 6-13: Comparison of cytoplasmic versus nuclear % LP cells stained with Snail1

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.3.C Twist

Matrix was devoid of Twist staining in LP area. There was no difference in % cell staining (all around 20%) between groups but a shift from cytoplasmic to nuclear staining in the COPD group, especially in COPD-Ex smokers ($P<0.05$) (Figure 6.6 and 6.14). In addition, nuclear to cytoplasmic ratio was also observed to be high in two above mentioned clinical groups ($P<0.05$).

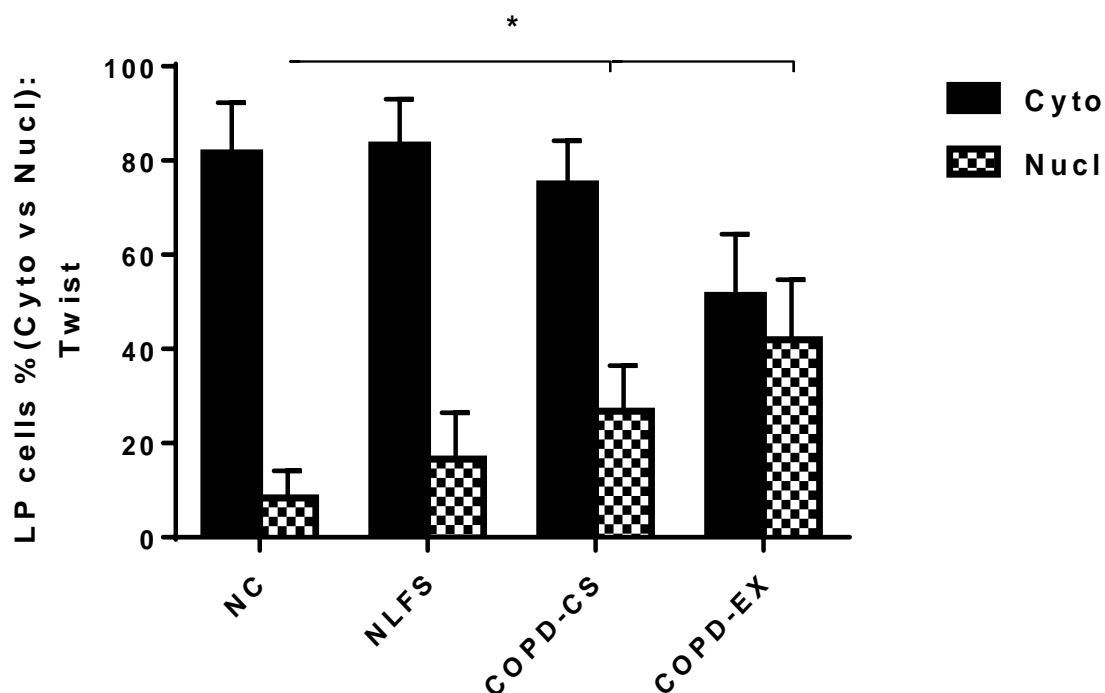


Figure 6-14: Comparison of cytoplasmic versus nuclear % LP cells stained with Twist

between healthy non-smokers (N-C), smokers with normal lung function (NLFS), current smoking COPD (COPD-CS) and ex-smokers with COPD (COPD-EX).

6.3.4 Regression Analyses

The relationships between β -catenin and Snail1 cell expressions, each independently with lung function, TGF β 1-Smad system and EMT activity (as S100A4 expression), were quite similar in all compartments (i.e. for basal cells, Rbm cells and LP cells); relationship for Twist were weaker though still generally significant. In general, higher the expressions of the transcription factors were annotated with greater degrees of EMT activity and also with greater levels of airflow obstruction. These relationships were strongest in basal cells and in COPD-CS, and representative regressions, are shown only for this group (Figure 6.15, 6.16 and 6.17).

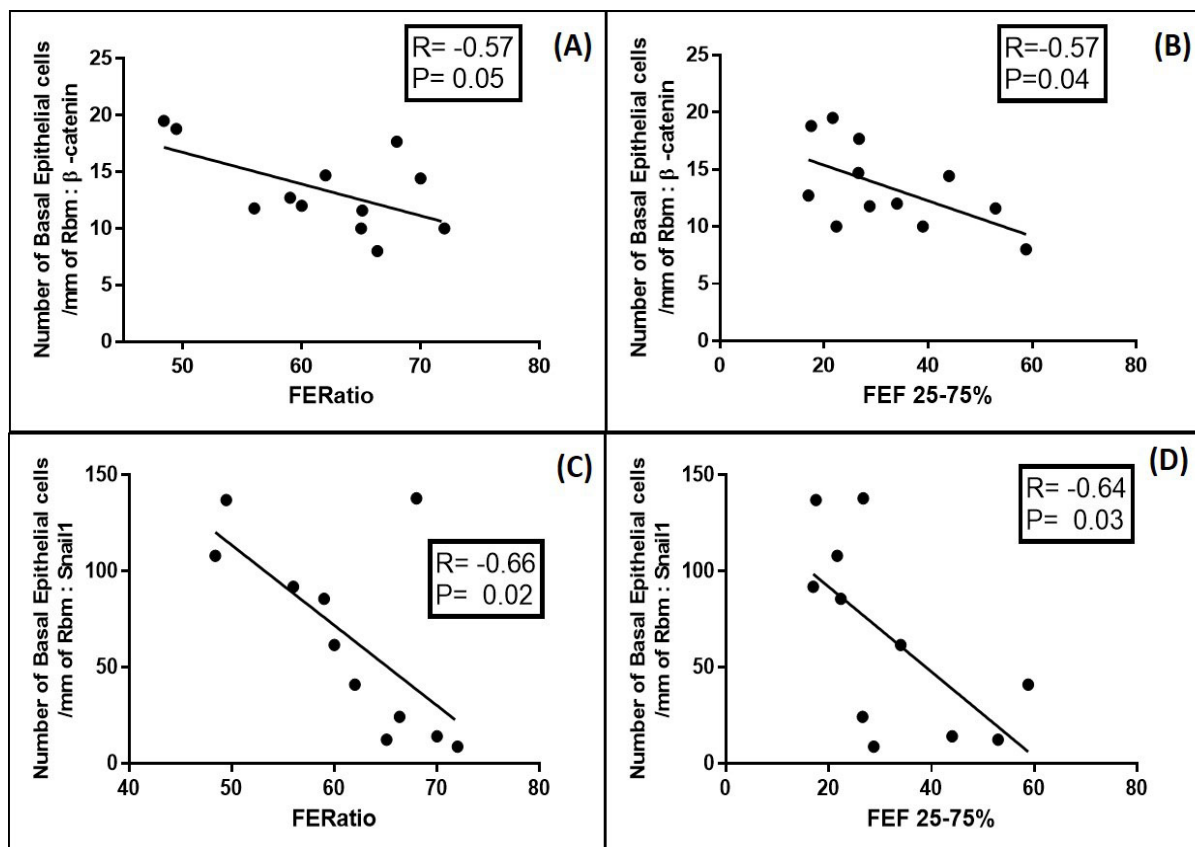


Figure 6-15: Regression Analysis of Transcription Factor Clusters and Lung Function test

A) Correlation between number of basal epithelial cells positive for β -catenin and forced expiratory ratio (FER); (B) The same for % predicted FEF₂₅₋₇₅ (an index of small airway calibre); (C) Correlation between Snail1 positive basal epithelial cells and forced expiratory ratio (FER); (D) Correlation between basal epithelial cells positive for

Snail1 with FEF 25-75% predicted. All these correlations are from the current-smoking COPD group (COPD-CS).

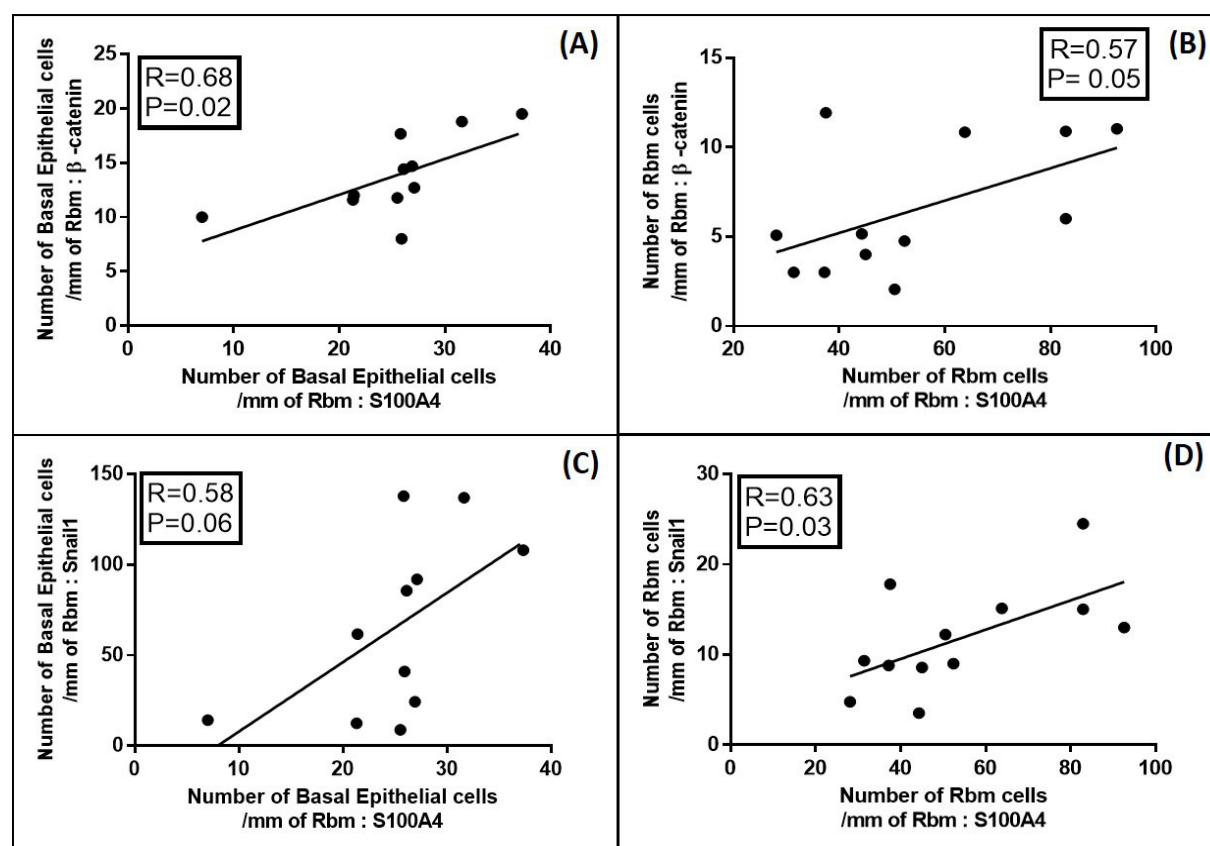


Figure 6-16: Regression Analysis of Transcription Factor Clusters and S100A4

(A) Correlation between number of basal epithelial cells positive for β -catenin with S100A4 positive basal epithelial cells; (B) Correlation between number of Rbm cells expressing β -catenin with S100A4 positive Rbm cells; Similarly, (C) Correlation between number of basal epithelial cells positive for Snail1 with S100A4 positive basal cells; (D) Correlation between Rbm cells expressing Snail1 with S100A4 positive Rbm cells. All these correlations are also from the current smoking COPD group (COPD-CS).

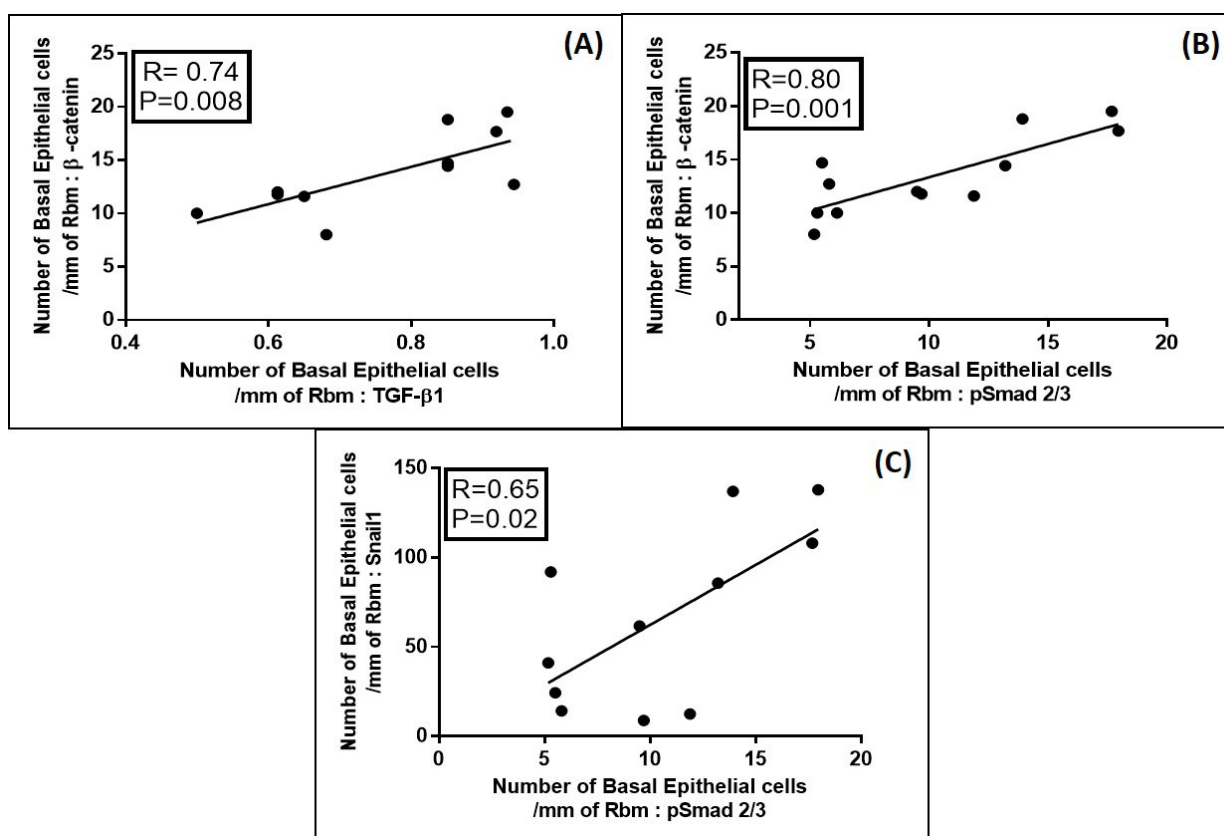


Figure 6-17: Regression Analysis of Transcription Factor Clusters and TGF β -Smad system

(A) Correlation between number of β -catenin positive basal epithelial cells with TGF β_1 positive basal epithelial cells; (B) Correlation between number of basal epithelial cells positive for β -catenin with pSmad 2/3 positive basal cells; (C) Correlation between basal epithelial expressing Snail1 with pSmad 2/3 positive basal epithelial cells. All these correlations are also from the current smoking COPD group (COPD-CS).

6.4 Discussion

I discussed in chapter 5 my data in regard to the TGF β 1-Smad pathway in COPD, and its relationships with both EMT and airway obstruction. I have now addressed similar issues with the classic pro-EMT β -catenin-Snail1-Twist transcription factor cluster, usually thought to be activated by the Wnt system through cell surface Frizzled receptors (Fodde & Brabletz 2007). I have now provided strong evidence for a general up-regulation of this system in smokers and once again especially in COPD. A particular feature was marked shift in these transcription factor expressions from the cell cytoplasm (and in the case of β -catenin first from the cell membrane) into the cell nucleus. This was evident not only in the epithelial basal cells but also in the hyper-cellular reticular basement membrane (Rbm) and to an extent in lamina propria stromal cells. There were strong generalised associations between the expression of these transcription factors and EMT activity (using expression of the classic mesenchymal marker S100A4) and notably also airflow obstruction.

Although several studies have demonstrated a role of the canonical Wnt/ β -catenin signalling pathway in fibrosis and tissue remodelling (Cheon et al. 2004; Chilosì et al. 2003; Clevers 2006), little has been known about how β -catenin may be involved in the pathology of COPD. My observations do consolidate the recent report of Wnt up-regulation in COPD airways and its induction in cultured airway cells by cigarette smoke (Heijink et al. 2016). In addition, various growth factors, including TGF- β 1, can activate β -catenin signalling either directly or via autocrine Wnt ligand production (Cheon et al. 2004; Guo & Wang 2009; Nunes et al. 2008) and this would fit with my observation of a significant cross-association with the TGF β 1 and pSmad 2/3 pathways in these human tissue studies.

Stabilized (non-phosphorylated) β -catenin activates several target genes including those for MMP's, growth factors, ECM proteins and pro-inflammatory mediators and enzyme (Clifford, Deacon & Knox 2008; Doyle & Haas 2009; Rahmani et al. 2005) and most importantly has been postulated as a key inducer of EMT in several tissues (Nagarajan et al. 2012). In addition, Baarsma et al observed that β -catenin expression is higher than normal in primary pulmonary fibroblasts from COPD subjects (Baarsma, H. A. et al. 2011), which may reflect my findings of expression by minority of LP stromal cells.

Snail1 and Twist have been implicated previously in induction of EMT in airways with protein and mRNA expression of mesenchymal markers and EMT-related transcription factors increased in cultured epithelial cells (Nishioka, M et al. 2015). The findings of the present study are novel in that I have not only shown a general increase in expression of transcriptional factors in all airway wall compartments, but also a downstream cytoplasmic to nuclear shift in smokers and especially in COPD. These changes were most evident in the basal epithelial cells in current smoker COPD. These findings have allowed us to develop a working model of COPD pathogenesis which can be tested by further experiment.

β -catenin and Snail1 and Twist are intimately interlinked because the majority of β -catenin's action is mediated through the other two transcription factors (Li, J & Zhou 2011; Morin 1999). Twist as an important transcriptional factors for fibrosis was first demonstrated in murine model of virus-induced lung fibrosis and in alveolar epithelial cells from lung tissue of idiopathic pulmonary fibrosis (IPF) patients (Pozharskaya et al. 2009). My present study suggest that these processes are also active in the airways in smoking-related COPD.

One of the most remarkable findings in my present study was the significant correlation of basal epithelial cell transcription factor expression with both an EMT activity marker and also airflow obstruction. This latter "mechanistic" relationship gives my findings clinical and potentially translational relevance. However, I also found a strong relationship in my previous study between Smad expression in the epithelium and airway obstruction (Chapter 5) though interestingly this was not found with TGF β 1 expression. This emphasise that factors other than this growth factor, are also likely driving EMT and down-stream airway fibrosis, making this a complex system unlikely to be amenable to an easy therapeutic intervention.

The strengths of the present study include the use of relevant human tissue in very well phenotyped individuals including mild to moderate COPD patients and comprehensive appropriate controls, and with sufficiently robust numbers giving sufficient power to detect these fascinating findings. I have focused on mild to moderate COPD patients, because active pathogenic mechanisms at this stage will be core ones, and not unduly influenced by later secondary complications such as

infection, inflammation and immune activation in airway lumen and without significant emphysema to affect airflow.

There are also some limitations to this study. Firstly, it has not undertaken comprehensive longitudinal analysis which could relate variable transcriptional factors expression to individual disease progression. It is noteworthy, for example, that even in the normal smoker control group there were quite strong relationships between transcription factors and decreasing lung function, even if technically all within the non-COPD range, suggesting that there are individuals on the way to full-blown COPD; one could also speculate that they may be of particular risk of lung cancer. Secondly, I am not sure about the detailed phenotype of the LP cells expressing β -catenin/Snail1/Twist, although descriptively they seemed to be stromal cell and not immune/inflammatory. Double staining will be a future goal. Thirdly, my control subjects were somewhat younger than the smoker/COPD group, but transcription factor expression levels were not age related in any group; and finally, this study used large airway biopsies while the predominant anatomic site of airflow limitation is in the small airways. I did this because recruiting volunteers for bronchoscopic airway sampling allowed us access to fit subjects with well-defined phenotypes including COPD subjects with relatively mild disease without confounding drug treatment or the pathology present in resected lung tissues. Further, I know that although EMT is especially active in larger airways, it is also present in small airways and lessons learned in one site are likely to reflect also what is happening throughout the airway tree; it is telling that both EMT activity (Chapter 4) and these current transcription factor expressions were strongly related to small airway function (FEF_{25-75%}). Even so, it is now certainly well worth the effort to try and replicate these finding in small airway samples.

In conclusion, I observed that the β -catenin/Snail1/Twist transcription factor cluster is activated in smokers and especially in COPD, and that their expression is remarkably closely related to both EMT activity and airway obstruction. I feel that this work is opening up novel understanding of the fundamental mechanisms involved in COPD patho-physiology. It will also open a new horizon in research for therapeutic targets in COPD patients, especially perhaps those who are in their early stage of COPD, and indeed more generally in smokers who are vulnerable to development of COPD.

Chapter 7

7. Epithelial mesenchymal transition (EMT) and non-small cell lung cancer (NSCLC): A mutual association with airway disease

7.1 Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death worldwide (Siegel, R., Naishadham, D. & Jemal, A. 2013). Among NSCLC variants, adenocarcinoma is the most common histological subtype. (40% of cases), while squamous cell carcinoma is the second most common type (approx. 25-30%). Surgical resection is the treatment of choice for early-stages though recurrence and metastasis are the common even after resection (D'Amico 2008; Williams et al. 2006). Since metastasis is the main obstacle for long-term survival after surgical resection, identification of prognostic molecular markers early on, related to aggressiveness, would be clinically useful.

For metastatic disease, it is widely accepted that invasion of extracellular matrix, vascular dissemination, and homing of cancer cells are major steps (Duffy, McGowan & Gallagher 2008). However, tissue invasion, is highly dependent on the structure of the primary organ and seems mechanically difficult due to lack of anatomic lung structures that can serve as routes for invasion. Therefore, in NSCLC, invasion needs newly formed desmoplastic stroma, containing active fibroblasts and a dense network of collagen and elastin, embedded in a ground substance, composed of proteoglycans and glycoproteins (Liotta 1986). Sakurai and colleagues tried to grade this infiltration process in NSCLC histopathologically by the degree of invasion and showed that grade was related to prognosis (Sakurai, H et al. 2004). Travis and colleagues further showed that breaking through epithelial basement membrane is also of major prognostic significance (Travis & Harris 2004).

Invasion into the surrounding tumorigenic desmoplastic stroma is enhanced by epithelial-mesenchymal transition (EMT) activity in cancer cells. EMT is a cellular process of morphologic and functional trans-differentiation from an epithelial to mesenchymal phenotype which is also implicated in the conversion of early-stages into invasive malignancies (Chen, N et al. 2014; Iwano, M. et al. 2002; Kang, Y &

Massagué 2004; Yilmaz, Christofori & Lehenbre 2007).

As consequence of EMT, resultant mesenchymal cells, digesting through the basement membrane (Gotzmann et al. 2004; Gupta & Massagué 2006; Jechlinger et al. 2003; Lee, J. M. et al. 2006) activate a gene program characteristics of cells (Kang, Y & Massagué 2004; Klymkowsky & Savagner 2009). EMT has been shown to be active in a number of epithelial cancers, e.g. pancreatic cancer, gastric, and colorectal carcinomas (Alves et al. 2007; Bukholm, Nesland & Borresen-Dale 2000; Kang, H et al. 2011; Nishioka, R et al. 2010) and indeed epithelial cancers constitute the vast bulk of human malignancies. Thus, EMT is an important event in the progression, invasion and metastasis of carcinomas and related in general to a particularly dismal prognosis (Gupta & Massagué 2006; Lee, J. M. et al. 2006; Thiery, J. P. 2002).

In the present study, I have taken a somewhat new direction for human work by investigating the heterogeneity of expression of classic EMT-related proteins including S100A4, vimentin and N-cadherin, as well as epithelial activity marker EGFR and a vascularity marker, Type-IV collagen for endothelial basement membrane (Soltani, Wood-Baker, et al. 2012). I have divided NSCLCs virtually between central and peripheral components, assessed their individual grade of differentiation and also obtained their final recorded histopathological TNM staging. I have investigated whether the expression pattern of EMT bio-markers would be greater at the peripheral area 'leading edge' of NSCLC tumors compared with central areas, and correlate with tumour differentiation and staging in both (adeno and squamous cell carcinoma). Finally, I have assessed how EMT expression and vascularity in the tumors may relate to such changes in the large and small airway walls from corresponding individuals in the resected lung specimens.

7.2 Over view of materials and methods

I employed classical immune-histochemical methods to evaluate EGFR, EMT biomarkers (S100A4, vimentin, N-cadherin) and Type-IV collagen expression in the NSCLC from well phenotyped participants. Details of study participants are given in Table 7.1.

| Groups (numbers) | Adeno CA (n=15) | Squamous cell CA(n=10) |
|----------------------------------|-------------------|------------------------|
| Male/Female | 11/4 | 7/3 |
| Age (years) | 65 (55.7-68.7) | 54 (46-68.3) |
| Smoking pack years (Range) | 41 (25.2-44.3) | 35 (28.4-43.9) |
| <u>T factor</u> | <u>(n)</u> | <u>(n)</u> |
| T1a/1b | 8 | 6 |
| T2a/2b | 5 | 3 |
| T3 | 2 | 1 |
| <u>N factor</u> | <u>(n)</u> | <u>(n)</u> |
| N1 | 11 | 6 |
| N2 | 4 | 4 |
| <u>Vascular Invasion</u> | 3 (+) 12 (-) | 3 (+) 7 (-) |
| <u>Pleural invasion</u> | 2 (+) 13 (-) | 1 (+) 9 (-) |
| <u>Lymphatic invasion</u> | 3 (+) 12 (-) | 2 (+) 8 (-) |
| FEV1 % predicted | 82 (75-91.4) | 80 (72-87.4) |
| FEV/FVC %* | 66 (62.9-76.1) | 68 (60.9-73.1) |
| FEF25-75% predicted | 52.5 (39.9-75.1) | 53 (36-70.8) |

Table 7.1: Demographic detail and lung function data of NSCLC patients

Data expressed as mean and CI95%;

T= Tumor Size

N= Lymph node involvement

FEV1= Forced Expiratory Volume in 1 second

FVC =Force Vital Capacity

FEF = Force Expiratory Flow

7.3 Results

7.3.1 EMT biomarker expression in NSCLC

For both tumour types, and for all bio-markers, (EGFR for epithelial activation; S100A4, vimentin and N-cadherin, for EMT) and also for collagen-IV for vessels, expression of protein was more marked in the leading edge peripheral area than centrally, $P < 0.05$ for squamous cell carcinoma (Figure 7.1, 7.2 and 7.3) and $P < 0.01$ to $P < 0.001$ for adeno-carcinoma (Figure 7.4, 7.5, 7.6, 7.7 and 7.8).

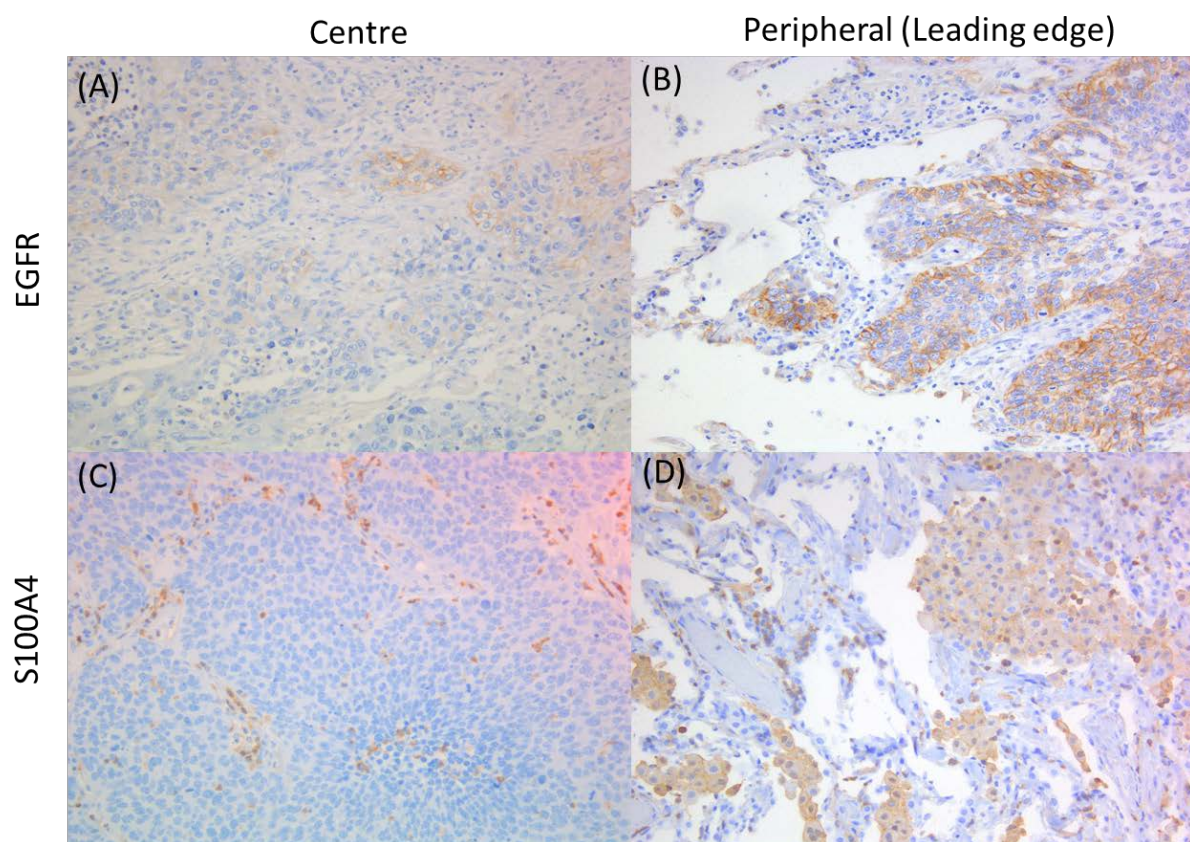


Figure 7-1: Representative photomicrograph of EGFR and S100A4 expression in squamous cell carcinoma

A) & C) Central Portion, B) & D) Peripheral leading edge. Original magnification, $\times 400$. Scale bar = 50 μm .

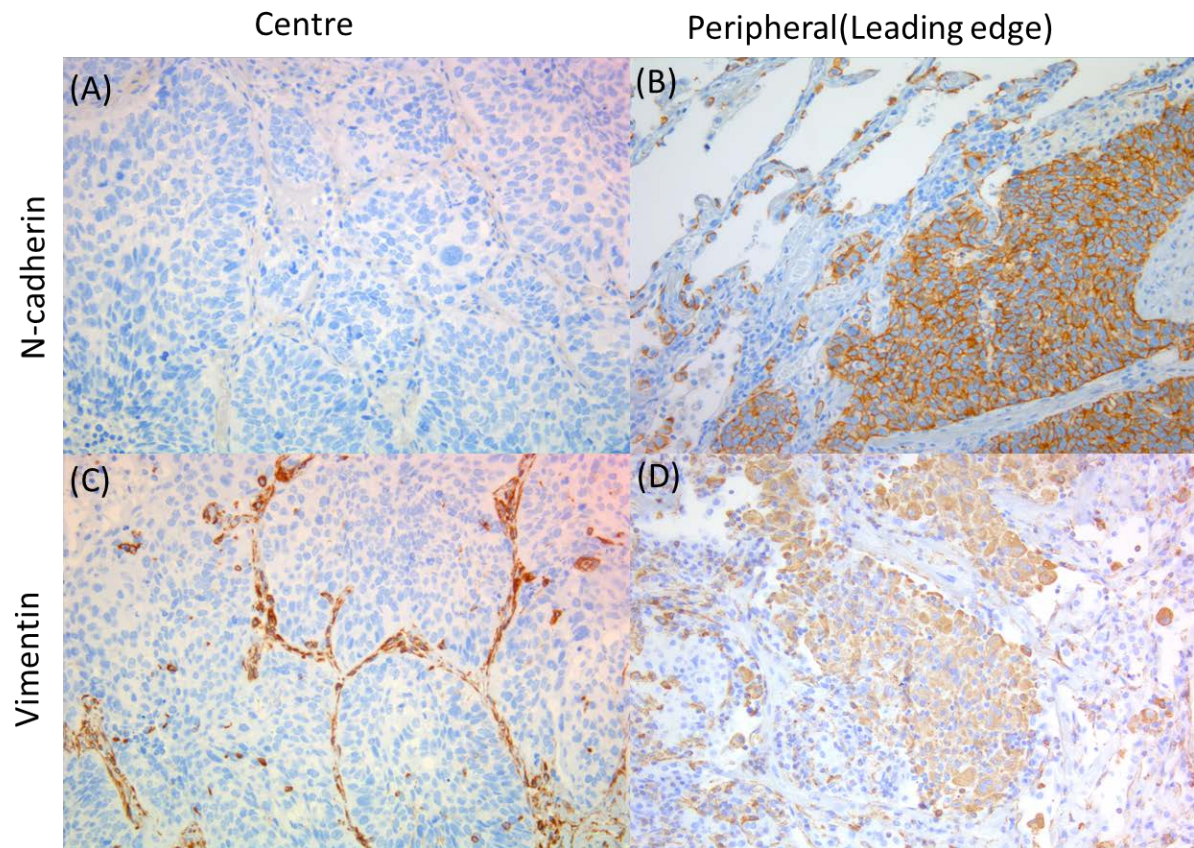


Figure 7-2: Representative photomicrograph of N-cadherin and vimentin expression in squamous cell carcinoma

A) & C) Central Portion B) & D) Peripheral leading edge. Original magnification, $\times 400$.

Scale bar =50 μm .

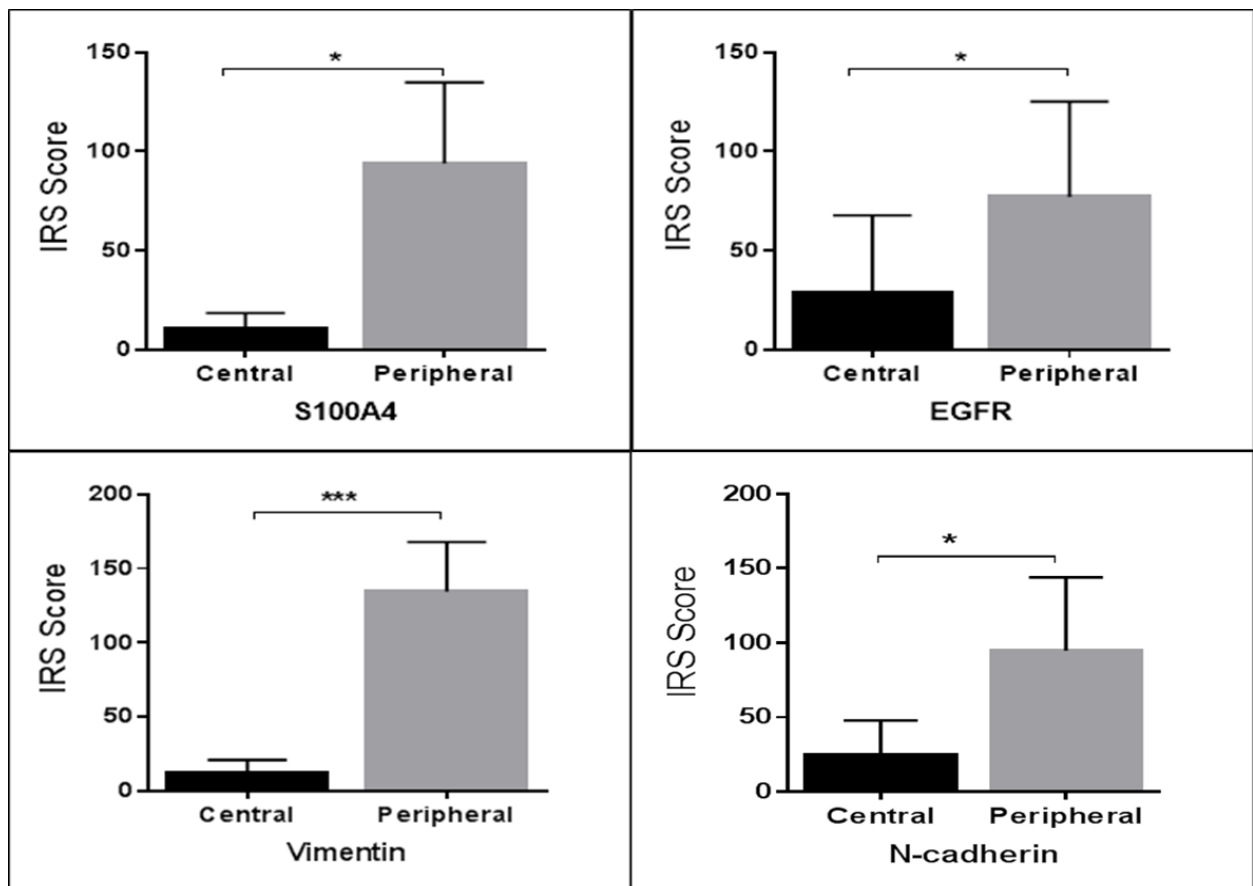


Figure 7-3: Comparison between central and peripheral leading edge of squamous cell carcinoma

(A) S100A4, (B) EGFR, (C) vimentin and (D) N-cadherin expression (IRS score).

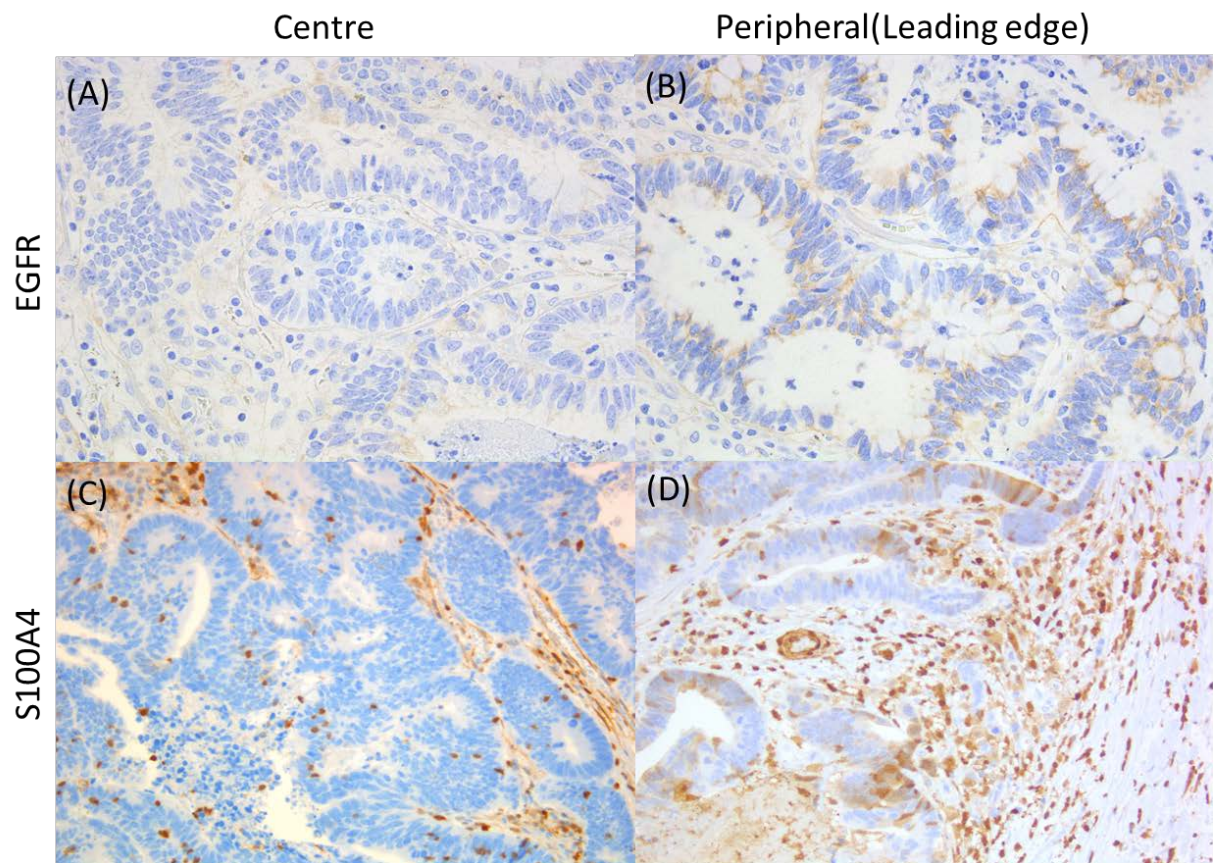


Figure 7-4: Representative photomicrograph of EGFR and S100A4 expression in Adeno carcinoma.

A) & C) Central Portion, B) & D) Peripheral leading edge. Original magnification, $\times 400$.
Scale bar =50 μm .

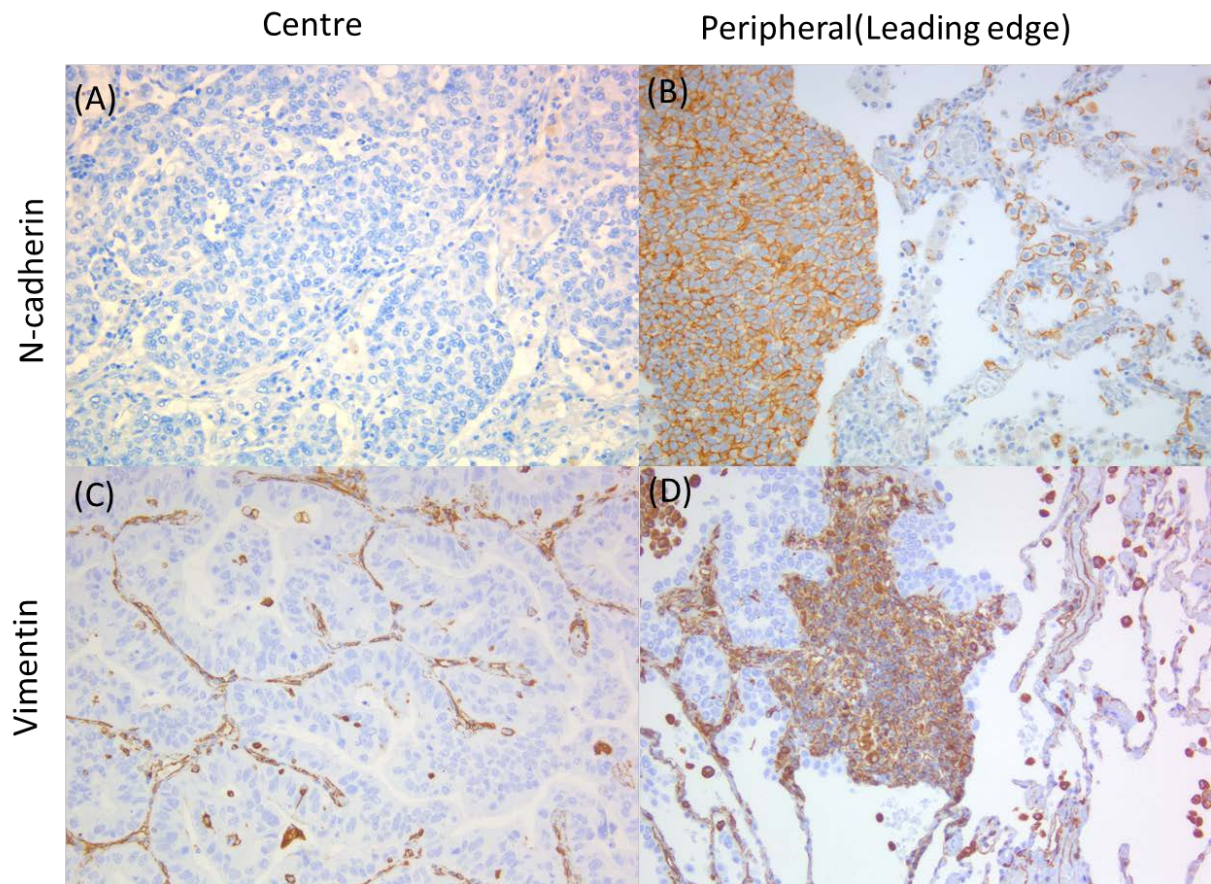


Figure 7-5: Representative photomicrograph of N-cadherin and Vimentin expression in Adeno carcinoma

A) & C) Central Portion, B) & D) Peripheral leading edge. Original magnification, $\times 400$.
Scale bar =50 μm .

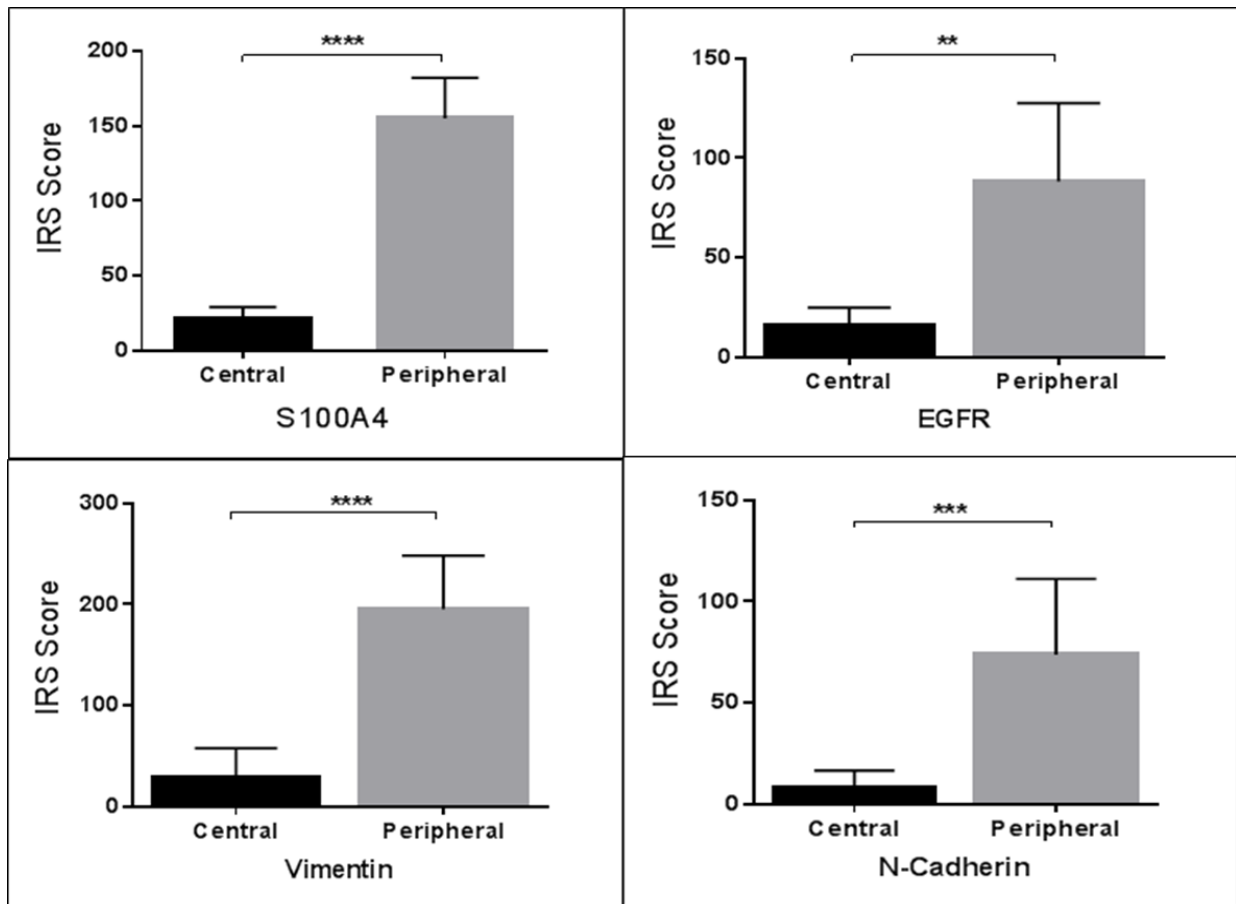


Figure 7-6: Comparison between central and peripheral leading edge of Adeno carcinoma

(A) S100A4, (B) EGFR, (C) vimentin and (D) N-cadherin expression (IRS score).

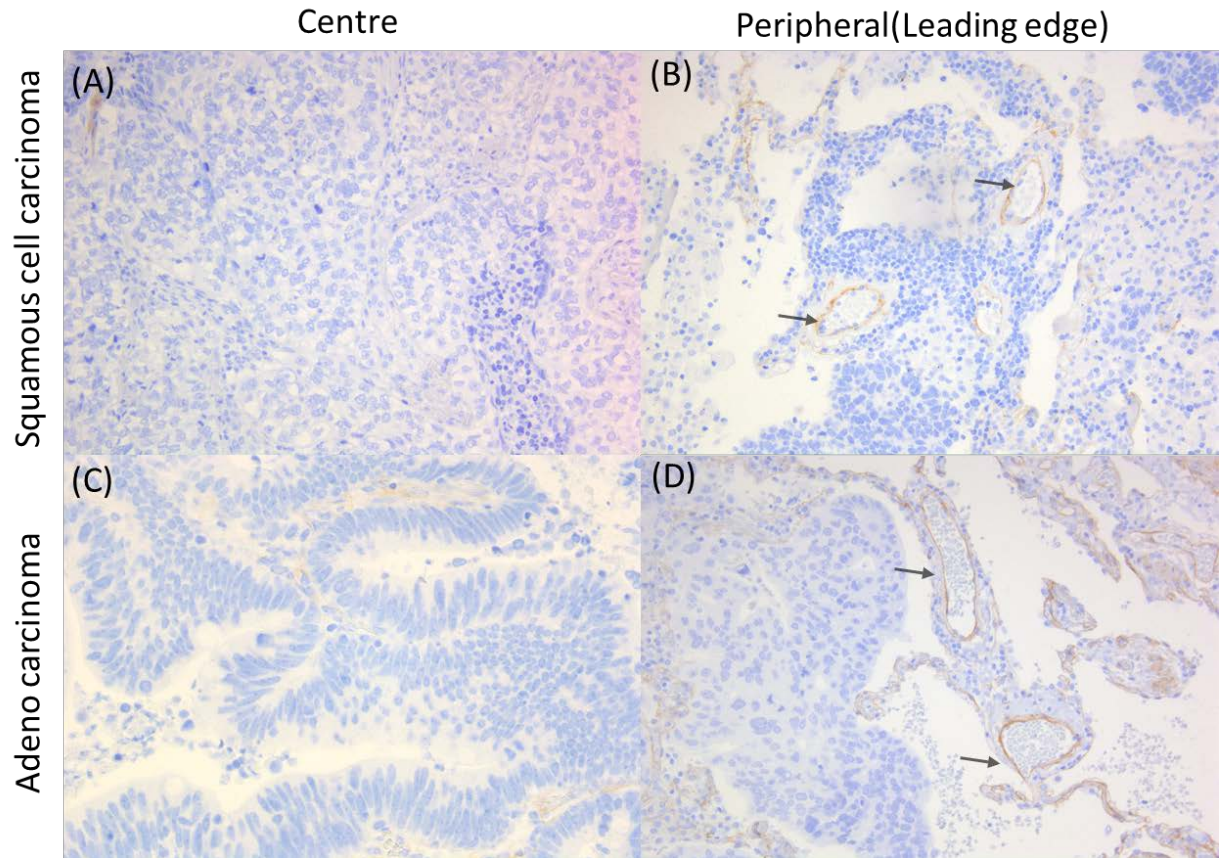


Figure 7-7: Photomicrographs of Type-IV collagen expressing blood vessels.

A & C) Central Portion, B & D) Peripheral leading edge. Original magnification, $\times 400$.

Scale bar =50 μm .

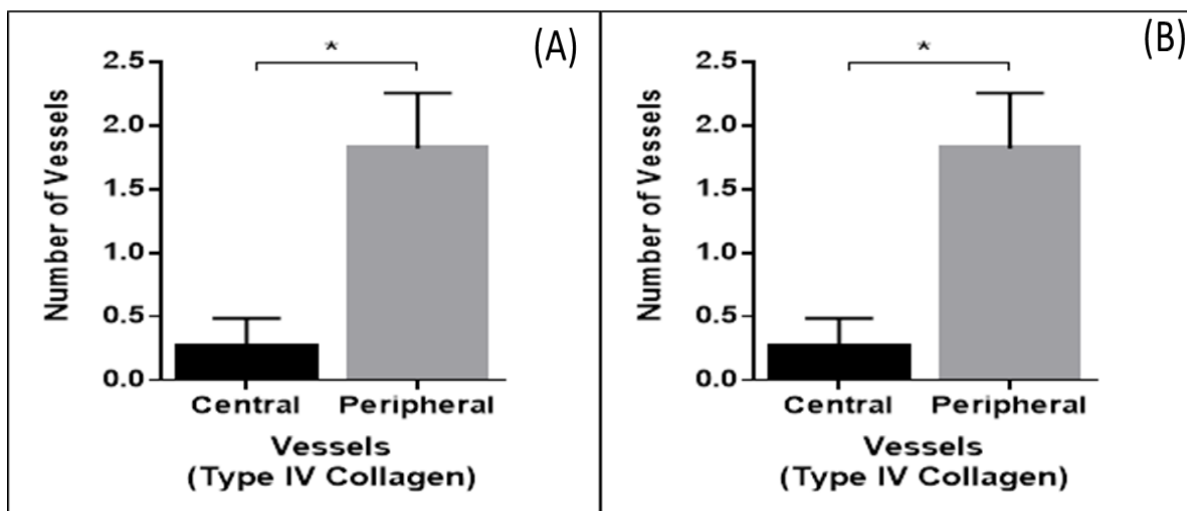


Figure 7-8: Comparison of number of Type-IV collagen expressing vessels

between centre and peripheral leading edge of (A) Squamous cell carcinoma and (B) Adeno carcinoma.

7.3.2 Regression Analyses

7.3.2.A Adenocarcinoma

For EGFR and S100A4, there were strong relationships, between peripheral tumour expressions (IRS assessment) and both TNM stage ($P<0.05$) and differentiation ($P<0.05$) (Figure 7.9).

For S100A4 and vimentin, peripheral tumour cell expression was positively correlated to corresponding small airway epithelial S100A4 ($P<0.05$) and vimentin ($P<0.02$) expression (Figure 7.10), but not to large airway expression. There were no relationships found between tumour vascularity and that in either the large or small airways.

7.3.2.B Squamous cell carcinoma

Again peripheral leading edge area expression of EMT biomarker (S100A4 and Vimentin) and EGFR (IRS assessment) positively correlated with more advanced stages ($P<0.01$) and poor differentiation ($P<0.05$) of tumors (Figure 7.9)

There were significant relationships found between tumour EMT bio-marker expression (by IRS assessment) and corresponding large and small airway wall EMT marker expressions (Figure 7.10). There was also a strong positive association between peripheral leading edge, vessel density and large airway epithelial reticular basement membrane (Rbm) vascularity ($P<0.05$) (Figure 7.11).

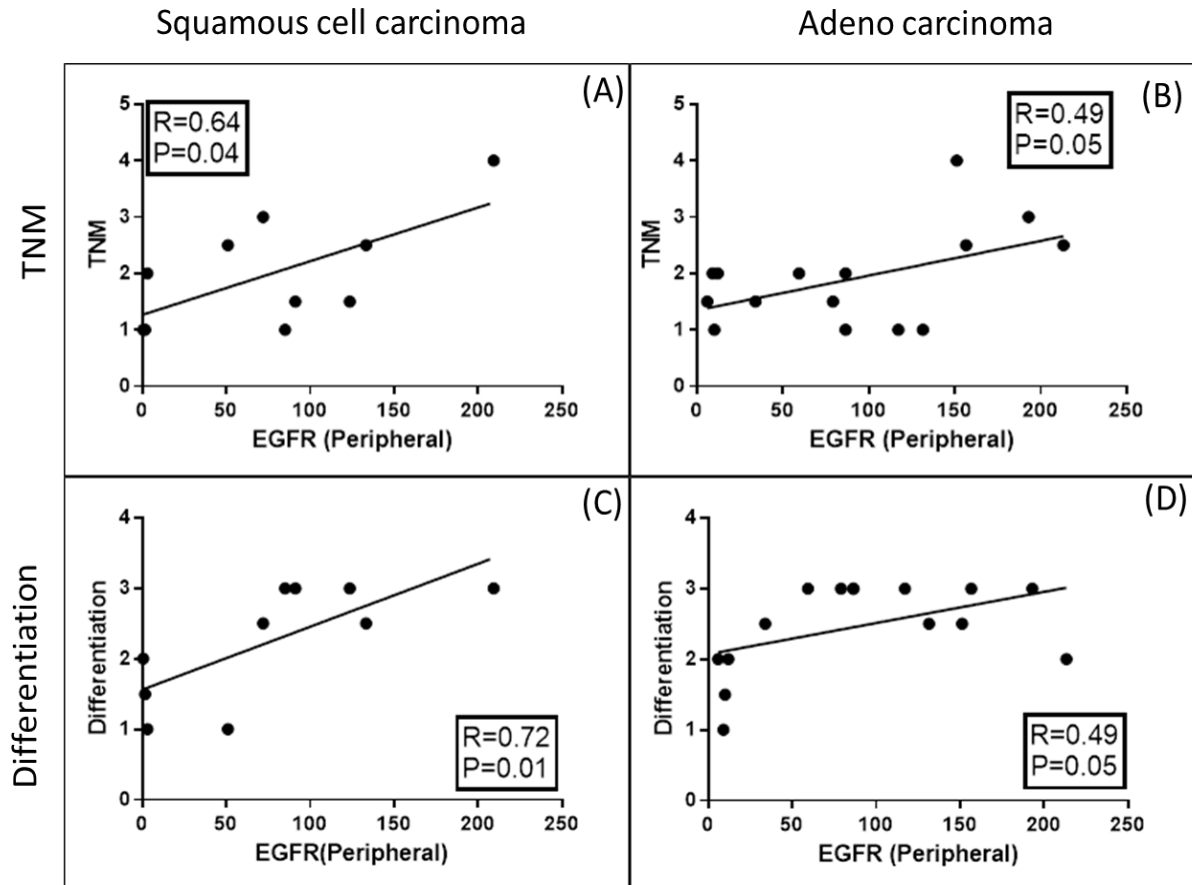


Figure 7-9: Regression Analysis of Tumor differentiation, TNM Stage and EGFR

A) Correlation between IRS score of EGFR at leading edge of Squamous cell carcinoma, and TNM stage (B) Correlation between IRS score of EGFR at leading edge of Adeno carcinoma, and TNM stage (C) Correlation between IRS score of EGFR at leading edge of Squamous cell carcinoma, and differentiation (D) Correlation between IRS score of EGFR at leading edge of Adeno carcinoma, and differentiation.

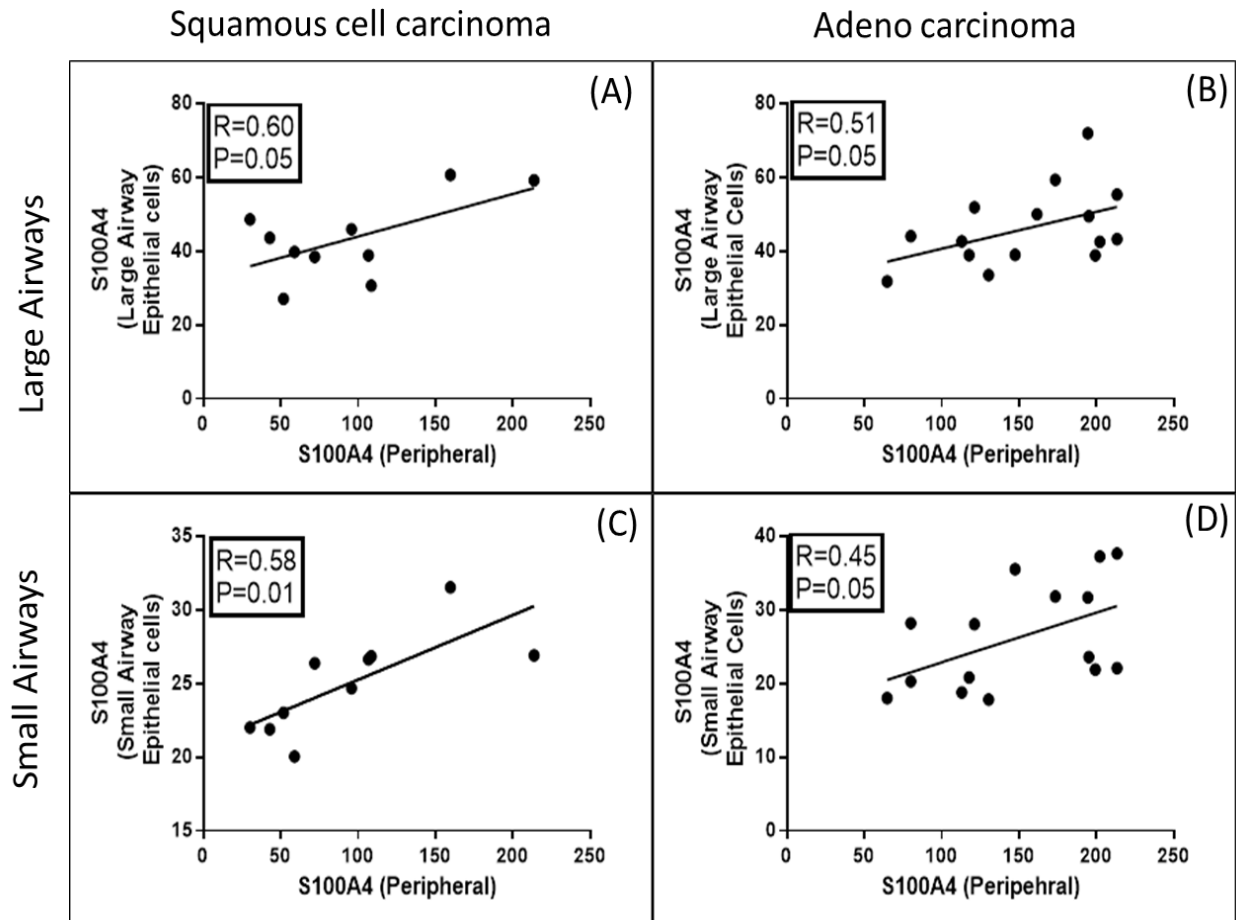


Figure 7-10: Regression analysis for S100A4 at tumour leading edge and in airways.

A) Correlation between S100A4 (IRS score) at leading edge of Squamous cell carcinoma, and number of basal epithelial cells per mm of Rbm, positive for S100A4, in large airway (B) Correlation between S100A4 (IRS score) at leading edge of Adeno carcinoma, and number of basal epithelial cells per mm of Rbm, positive for S100A4, in large airway C) Correlation between S100A4 (IRS score) at leading edge of Squamous cell carcinoma, and small airway's number of basal epithelial cells per mm of Rbm, positive for S100A4 (D) Correlation between S100A4 (IRS score) at leading edge of Adeno carcinoma, and small airway's number of basal epithelial cells per mm of Rbm, positive for S100A4.

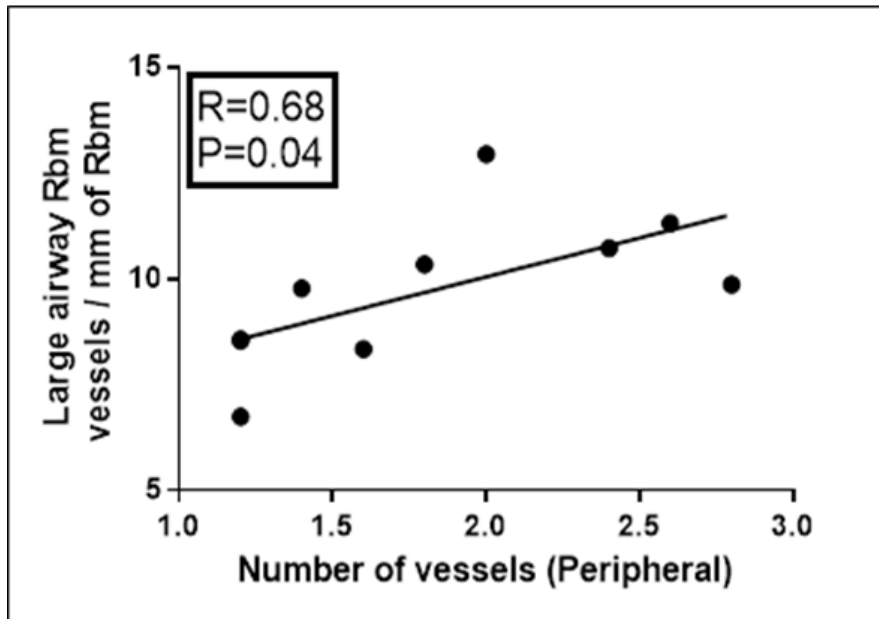


Figure 7-11: Regression analysis for number of blood vessels at tumour leading edge and in airway.

Correlation between number of Type-IV collagen positive vessels at leading edge and number of Rbm vessels per mm of Rbm, in large airways.

7.4 Discussion

Non-small cell lung cancer (NSCLC) is the most predominant type of lung cancer and the leading cause of cancer deaths worldwide (Jemal et al. 2010). However, NSCLC has been relatively little studied for the potential importance of EMT in determining its biology and outcomes. I have now shown that there is obvious heterogeneity in central and peripheral leading edge of NSCLC (adeno and squamous cell-carcinoma), not only in terms of EMT biomarker expression (S100A4, Vimentin and N-cadherin) and EGFR expression but also in vessel density as well. In addition, there was a strong correlation between EMT biomarker expression in the leading edge and with advanced stage and poor differentiation of NSCLC. There was also a positive correlation between leading edge EMT expression in both tumour types and airway epithelial EMT activity, and for squamous cell-carcinoma only between peripheral tumour and large airway Rbm vascularity.

The current literature has included a focus on gene deletions and insertions in exon 19 and point mutations in exons 18 and 21 in the epidermal growth factor receptor (EGFR) (Otto et al. 2012; Uruga et al. 2010), expression of onco-fetal protein IMP3 (Beljan Perak et al. 2012) and specific gene promoter methylation (Ji et al. 2011) as prognostic markers in NSCLC. However, there has been little work solely on metastasis-related markers including EMT related proteins. Some studies have highlighted that centrally located tumour cells stained more positively for epithelial markers, but was absent at the invasive front of the tumour in lung cancer (Chiou et al. 2010; Hung et al. 2009; Tischler et al. 2011), but did not look at the inverse, i.e. more mesenchymal tumour cell expression at the leading edge. Both lower epithelial marker and higher mesenchymal marker expression suggest active EMT.

As EMT activity is considered to be one of the causes of morphological heterogeneity in general (Bartis et al. 2014) and now I observed in NSCLC that S100A4 and vimentin and N-cadherin expression was relatively low in the central region and so was blood vessel density, in comparison with leading edge, peripheral part. In the central area of NSCLC, the cells usually form irregular-shaped nests associated with marked desmoplastic stroma production (Chen, Z et al. 2014). In comparison, in the peripheral areas of primary NSCLC, the tumour cell infiltrate fills and destroys the alveolar spaces, but is associated with a weak desmoplastic reaction (Funai et al. 2003;

Watanabe et al. 2011). This latter area is also where I observed increased S100A4 and vimentin expression and an associated increase in tumour blood vessels. My observations are possibly in contrast to the study by Udagawa and colleagues who observed expression of molecular markers (EGFR, S100A4, CD44 and E-cadherin) at both peripheral and central area of squamous cell-carcinoma (Udagawa, H. et al. 2015).

The current study clearly showed that the expression level of N-cadherin was significantly higher at the tumour periphery than in the central area of NSCLC of both sorts studied. In digestive tract cancers (Lee, S-J et al. 2013) and oral squamous cell carcinoma (Wang, X et al. 2009), a decrease in E-cadherin expression was found at the invasive front of the tumor but they did not look at the “flip-side” expression of N-cadherin; a switch from E-cadherin to N-cadherin is a strong bio-marker of EMT (Gravdal et al. 2007).

As described earlier, EGFR is an example of a receptor tyrosine kinase (RTKs), a family of transmembrane proteins that serve as receptors for many growth factors, which then activate signalling pathways leading to cell proliferation and anti-apoptotic activity. Heterogeneous expression of RTKs in cancer cells is well recorded (Andersson, J et al. 2004) and in NSCLC heterogeneous EGFR expression within the same was reported (Grob et al. 2013; Italiano et al. 2006). Compatible with this, in my study, EGFR expression was significantly higher in the peripheral area than in the central area of the primary NSCLCs. However, Udagawa et al observed EGFR expression to be greater in the central tumour area than peripherally (Udagawa, H. et al. 2015), though this study dealt only with squamous cell-carcinoma. A retrospective analysis of the FLEX study suggested that chemotherapy with an anti-EGFR antibody, improved the overall survival of patients with tumours showing high EGFR expression, but not in those showing low EGFR expression (Pirker et al. 2012). It might be that early assessment of lung cancer for leading-edge EGFR expression could be especially useful for deciding on anti-EGFR therapy.

I also found strong relationships between EMT activity and both small and large airway EMT activity in squamous cell tumours though in adeno-carcinoma there was such a relationship only with EMT expression in small airways. Further, there was a strong association between peripheral squamous cell carcinoma vascularity with large airway

Rbm vascularity. This positive association is in agreement to my previous finding in Chapter 4 where Type-3 EMT (hype vascular Rbm) is thought to be a pro-malignant features in large airways. This was not the case for adeno-carcinoma.

These similarities with airway regions of likely origin, i.e. squamous cell-carcinoma from large airways and adeno-carcinoma from small airways suggest that those local environments are important for development of specific cancer types and for imprinting on the local EMT-related features which then influence tumour aggressiveness. This suggests that sampling of the airway wall to assess EMT and vascularity in large airway could be useful prognosticating, especially for squamous cells.

The strengths of the present study included the use of relevant human tissue in well phenotyped individuals, the inclusion of both adeno and squamous cell carcinoma patients and the sufficiently robust numbers giving sufficient power to detect these fascinating findings. We also focused on staging and differentiation of tumours, in order to be able to relate tumour characteristics to these important clinical factors. There were also some limitations to this study. Firstly, it was cross-sectional at a single time point and lacked the potential strength of a longitudinal study with early tumour assessment that could be related prospectively to out-comes, and although difficult, this should now be done in a replication cohort.

In conclusion, EGFR and EMT-related protein expression was markedly high at the peripheral leading edge of NSCLCs and related to tumour characteristics associated with poor prognosis. These changes could be potentially useful as early prognostic bio-markers. There were also interesting relationships between EMT-related tumour bio-marker expression and those in the corresponding airway epithelium and Rbm, which means that background airway changes may also be of clinical utility in tumour assessment.

Chapter 8

8. Summary and Conclusions

8.1 Overview of results

There is emerging evidence that EMT and its associated pathways plays a key role in the pathogenesis of both smoking-related COPD and COPD-related lung cancer. My thesis reports novel findings from investigations of EMT in human lung tissues, especially airway epithelium, Rbm and LP collected from controls, normal smokers and COPD patients, either from endobronchial biopsies or from resected lung tissue. I proposed that increased expression of EMT-related biomarkers and related downstream transcription factor clusters/pathways are major pathological changes underlying airway remodeling and potentially lung cancer in COPD patients. These changes in the airways have not been described so comprehensively in the literature before, and I believe this thesis will significantly add to the current understanding of the pathogenesis and patho-physiology of COPD, and will allow potentially new therapeutic approaches to emerge.

My thesis is divided into seven main sections. The first two chapters outline the current evidence for expression of EMT and relate downstream transcriptional factors/pathways in COPD. Third chapter details the demographics of study patients and methodological approaches employed. There are then four main results chapters with individual discussions.

In **Chapter 4**, I extended the evidence for airway EMT activity to the small airways in smokers and COPD compared to appropriate controls (normal lung tissue from transplant donors -unused), compared them to large airway findings, and related EMT activity to physiological abnormalities i.e. airflow obstruction in the same individuals.

In **Chapter 5**, I demonstrated enhanced expression of TGF β 1 and its canonical downstream Smad pathway (including activated pSmad 2/3 and 7) in large airway samples, again in well phenotyped COPD subjects and appropriate smoking and non-smoking controls. The increased pSmad 2/3, but not the TGF β 1, was significantly correlated with EMT activity and also airflow obstruction. This diversity of outcome suggested that factors other than or as well as TGF β 1 are driving this system.

In **Chapter 6**, I explored up-regulation of the β -catenin, Snail1 and Twist transcriptional factor cluster and also their cellular localization (membranous, cytoplasmic or nuclear) according to phenotype group in large airways. Not only were these transcription factors (TFs) generally up-regulated in COPD but there was a consistent shift of these TF towards more nuclear expression. In addition, expression of all these TFs (though weakest for Twist) was significantly correlated with EMT activity, the corresponding TGF β -Smad data and importantly once again airflow obstruction. This latter finding especially gives major functional relevance to these research outcomes.

Finally, in the last results chapter, **Chapter 7**, I demonstrated a substantial and highly significant difference in EGFR and EMT biomarker expression between central and peripheral leading edge (marked increase) in resected NSCLC (adeno- and squamous cell-carcinoma). In regression analysis, I also showed the expressions of all analyzed bio-markers were positively related to tumor TNM stage and differentiation score. Further, there were significant relationships between EMT activity at the peripheral leading edge and EMT expression in the corresponding non-tumorous airways, and for squamous cell-carcinoma a relationship between vascularity at the leading edge and vessel density in the Rbm in the corresponding large airways. This is the first direct evidence that EMT and EMT related angiogenesis in the airway wall may be imprinted on the biology of the tumor arising in those airways, and may provide a mechanism for the known relationship between COPD and lung cancer. These findings could be potentially useful as early prognostic bio-markers in lung cancer. Even sampling the corresponding airway wall may be useful in giving some indication of the likely aggressiveness of the tumor which has arisen from it.

Figure 8.1 sums up a lot of my data, enhanced by some of the facts we already knew about EMT-induction mechanisms. The overall work done has been related to airway function specially in COPD and with lung cancer as well. It would now be interesting to investigate whether current therapeutics in COPD may affect these pathways as a means by which they seem to stabilize the natural history of COPD, as well as exploring how newer anti-fibrotic drugs being introduced for treatment of IPF may affect these pathways in the airways as well as the lungs.

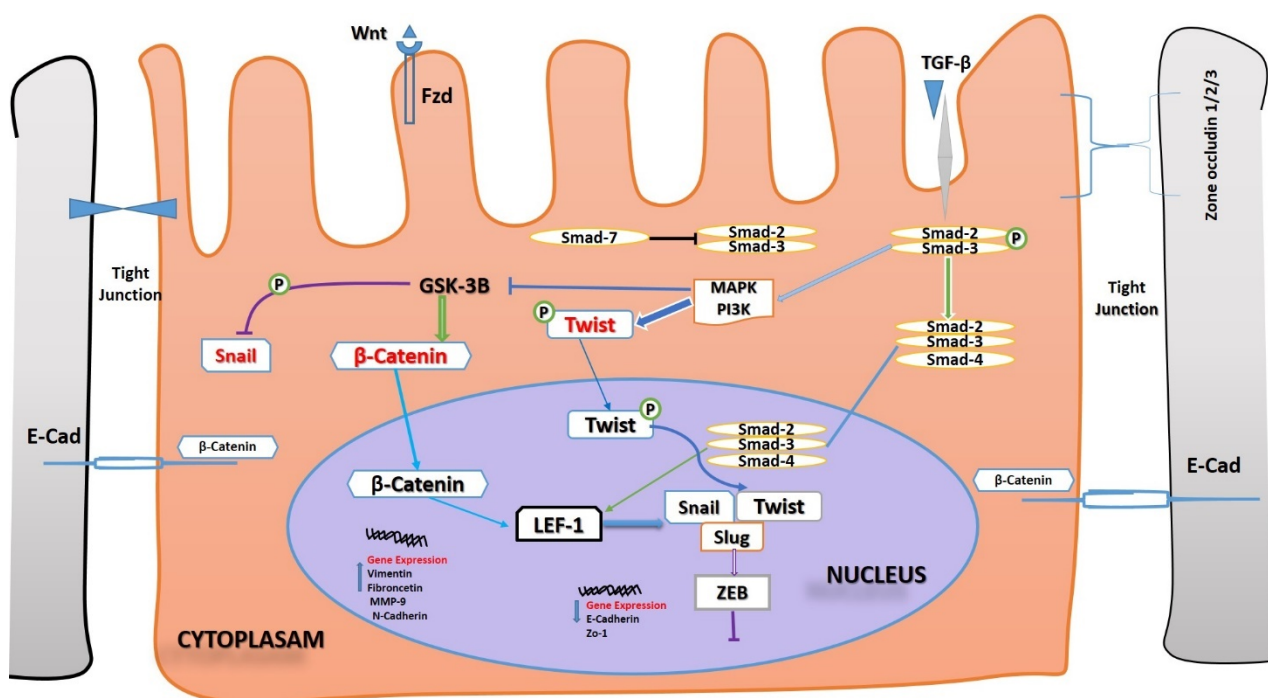


Figure 8-1: EMT associated transcriptional factors/pathways, as potential therapeutic targets.

As part of this final over-view of my thesis, perhaps two extra but important points need to be made:

Firstly, all my work was in human tissue and so has important limitations; namely, that it is difficult to manipulate the situation and likewise to do longitudinal studies, although our group does have a history of managing to do this in volunteer clinical groups in spite of the difficulties involved in undergoing multiple bronchoscopies (Sohal et al. 2014; Walters, Reid, et al. 2008; Walters, Soltani, et al. 2008). Thus, although our

phenotypic groups were very well characterised, the difference between groups that we found are likely, but not necessarily functionally and causatively related to these phenotypes. Ideally, this sort of human work should be done as part in an extended multi-modality team, where hypotheses generated can be tested across in-vitro studies in primary or secondary human cell culture, and also in animal models. Both of these latter modalities would then allow for manipulations e.g. smoke exposure and also suppression of key pathways and transcription factors etc. so as to be able to move from only making extrapolations and assumptions about the meaning of associations, to being more sure about cause and effect. However, we did not have these capacities available at the right time for me to exploit, and I would not have had the time in 3 years to cover all the ground I have achieved, if also pursuing such a multi-modality approach I had to do the work myself.

Thus practically to take such a multi-modality approach, one would need ready and committed collaborators. Our group did set up a cell culture facility 3 years ago, but much time has been needed to do extensive and mainly methodological evaluations, and data related directly to COPD and smoke exposures are only now being generated. We did not have the equipment or willing collaborators to do smoke exposure experiments in animals locally, although we do now have available extensive tissue from “smoked animals” from external collaborators, which will be part of the group’s next phase of this programme. A major problem with most smoke exposure studies in animals is that they are really about induction of innate inflammatory responses in the airway lumen (i.e. acute or sub-acute bronchitis) (e.g. Botelho et al. 2010) and not really a chronic disease process equivalent to airway-destructive COPD. Our group is currently evaluating a collaborator’s chronic exposure animal model to see how equivalent to relevant human airway disease this really is. If it is suitable, then we could be highly productive over the next few years in doing the sort of work needed to consolidate our findings to better “prove” cause-effect relationships.

Secondly, and perhaps in stark contrast to the above, working with human tissue from relevant patients is at the same time also a major strength of this work, especially the ability to relate our quantitated markers of EMT to transcription factors, and indeed both of these to clinically relevant physiological indices of the core disease process i.e. airflow obstruction. Few other centres in the world have been able to do this (Gohy et al. 2015; Wang et al. 2013). I believe that it is reasonable to say that this programme

of work from our group has put the idea on the collective respiratory research map that EMT may be indeed intimately related pathogenically to airway fibrosis and airway obstruction in COPD. A number of important papers that I have quoted which essentially replicate our finding, e.g. Gohy et al. refer to our work as their starting point. What my work to date has lacked, and it is a significant weakness, is the ability to use any intermediate measure of COPD airway pathology, and especially airway thickening, fibrosis and activation of sub-epithelial matrix-producing stromal cells, in regressions against my EMT-related end-points. Data on exactly these measures are now emerging from others in our team, and will allow us in the near future to do these analyses.

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